IN VITRO PROPAGATION AND SHOOT-TIP GRAFTING OF PATHARNAKH (*Pyrus pyrifolia* (Burm F.) NAKAI) PEAR

Dissertation

Submitted to the Punjab Agricultural University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSPHY
in
POMOLOGY
(Minor Subject: Agricultural Biotechnology)

By

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CERTIFICATE-I

This is to certify that the thesis entitled, **"*In vitro* propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burm F.) Nakia) pear"** submitted for the degree of Ph.D., in the subject of **Pomology** (Minor subject: **Agricultural Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Haseeb ur Rehman (L-2010-A-26-D)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE- II

This is to certify that the dissertation entitled "***In vitro*** **propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burm F.) Nakai) pear"** submitted by **Haseeb Ur Rehman (L-2010-A-26-D)** to the Punjab Agricultural University, Ludhiana in partial fulfillment of the requirements for the degree of Ph.D., in the subject of **Pomology (Minor subject: Agricultural Biotechnology)** has been approved by the Student's Advisory Committee after an oral examination on the same.

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The Beneficent, the Merciful, the All hearing, the All knowing
Owner of the day of Judgment.

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Abstract

In vitro propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burn F.) Nakai) pear was carried out in Tissue Culture Laboratory in Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-13. Nodal explants from Patharnakh (forced and active) and Kainth (forced and active) were used for their *in vitro* propagation protocol development. The effect of various media {1/2 MS (M_1), MS (M_2) and WPM (M_3)} and growth regulators (BAP, IBA and NAA) on establishment, proliferation and rooting of Patharnakh (forced and active) and Kainth (forced and active) was studied. Patharnakh scion derived from two sources, i.e. one derived from forced shoot tip (S_2) and another from *in vitro* shoot tip (S_1) having different lengths (L_1 = < 5mm, L_2 = 5-10 mm and L_3 = 10-15 mm) were used for shoot tip grafting on *in vitro* developed Kainth rootstock. Two grafting techniques viz, wedge (G_1) and horizontal (G_2) were used and shoot tip grafts obtained were allowed to grow in different media i.e. MS medium + 20 g/l sucrose (M_1), liquid medium composed of MS solution + 20 g/l sucrose (M_2), MS medium + 40 g/l sucrose (M_3) and liquid medium composed of MS solution + 40 mg/l sucrose (M_4). Observations on aseptic cultures (%) graft success (%), necrosis (%), vitrification (%) and vigour (1-3 point scale) were recorded. Besides this, pre-treatment of TDZ and 2, 4-D was given to scions before grafting by dip method for one minute. Observations regarding aseptic cultures (%), graft success (%), necrosis (%) and vitrification (%) were recorded. Necrotic culture (%) was found to be influenced by type of media and growth regulator fortification during establishment stage in both Patharnakh (forced and active) and Kainth (forced and active). Lowest necrotic culture percentage in Patharnakh (forced and active) was observed by using M_2 medium fortified with BAP (1.0 mgl^{-1}) and IBA (0.01mgl^{-1}) whereas, M_2 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved best by inducing least necrotic cultures in Kainth (forced and active). Maximum establishment (%) was obtained on M_3`medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) i.e 96.10 per cent in case of Patharnakh (forced) and on M_2 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) i.e 96.29 per cent in case of Patharnakh (active). Similarly, M_2 containing BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) gave maximum explant establishment of 52.80 and 63.60 per cent respectively in Kainth (forced) and Kainth (active). Maximum proliferated cultures in Patharnakh (forced) and Patharnakh (active) were obtained using M_3 medium fortified with BAP (2.5 mgl^{-1}) i.e 85.67 per cent and BAP (5.0 mgl^{-1}) i.e 79.47 per cent respectively. However, maximum proliferated cultures in Kainth (forced) and Kainth (active) were produced using M_3 medium supplemented with BAP (3.0 mgl^{-1}). Similarly the highest shoots per explant in Patharnakh (forced) and Kainth (active and forced) were obtained using M_3 medium supplemented with BAP (2.5 mgl^{-1}) and BAP (3.0 mgl^{-1}) respectively. However, maximum shoots per explant (3.08) in Patharnakh (active) were produced in M_2 medium supplemented with BAP (2.5 mgl^{-1}). Longest shoots in

Patharnakh (active) and Kainth (forced and active) were obtained in M_3 medium containing BAP (0.0 mgl^{-1}) i.e control. However, longest shoots (53.75 mm) in Patharnakh (forced) was obtained using M_3 medium containing BAP (0.5 mgl^{-1}). Rooting (%), roots per explant and root length was found to be influenced by type of medium and growth regulator fortification in both Patharnakh (forced and active) and Kainth (forced and active). Rooting (%) was maximum in Patharnakh (forced) i.e. 10.16 and Patharnakh (active) i.e. 9.16 using M_1 medium fortified with IBA (1.0 mgl^{-1}) while as maximum rooting (%) in Kainth (forced) i.e 13.34 and Kainth (active) i.e 14.08 was observed in M_1 medium supplemented with IBA (0.1 mgl^{-1}). Although no rooting was obtained in Patharnakh (forced and active) irrespective of media using NAA however, NAA (1.0 mgl^{-1}) induced rooting (%) of 29.61 and 27.46 using M_1 medium in Kainth (forced) and Kainth (active) respectively. Roots per micro-shoot were 2.38 and 2.60 respectively in Patharnakh (forced) and Patharnakh (active) using M_1 medium supplemented with IBA (1.0mgl^{-1}). Similarly in Kainth (forced) and Kainth (active), maximum roots per explant were obtained using M_1 medium supplemented with IBA (1.0 mgl^{-1}). NAA (1.0 mgl^{-1}) induced highest number of roots per explant in Kainth (forced), i.e. 3.40 and Kainth (active), i.e. 3.60 using M_1 and M_2 medium respectively. In Patharnakh (forced and active), maximum root length were obtained using M_3 medium supplemented with IBA (1.0 mgl^{-1}). However, M_3 supplemented with IBA (0.1 mgl^{-1}) resulted in maximum root length (31.15 and 31.10 mm) in Kainth (forced) and Kainth (active), respectively. NAA (0.1 mgl^{-1}) resulted in maximum root length (22.97 mm) in Kainth (forced) in M_1 medium, whileas in Kainth (active), NAA (1.0 mgl^{-1}) resulted in maximum root length of 23.22 mm using M_3 medium. Aseptic culture, graft success, necrosis, vitrification and vigour were found to be influenced by scion origin, scion length, grafting technique and medium used during shoot tip grafting. Aseptic cultures were maximum (84.34 %) by employing S_1, L_2, G_2 and M_1. Graft success was maximum (33.71%) by using S_1, L_2, G_1 and M_2. However, necrosis was least (0.0%) in many treatment combinations. Vitrification was also found to be influenced by various treatment combinations with least vitrification (12.01 %) by employing S_1, G_1, L_1 and M_4. Maximum vigour (2.68) was found by using S_1, G_1, L_2 and M_2. Pre-treatment with growth regulators was found to have beneficial effects on graft success and other parameters. TDZ (0.5 mgl^{-1}) + 2,4-D (5.0 mgl^{-1}) resulted in maximum aseptic cultures (88.78%), whileas highest graft success (42.34 %) was obtained by giving pre-treatment of TDZ (0.5 mgl^{-1}) + 2,4-D (7.5 mgl^{-1}). However, least necrosis (1.65 %) resulted by treatment of TDZ (0.75 mgl^{-1}) and minimum vitrification (4.11 %) was observed by treatment combination of TDZ (0.75 mgl^{-1}) + 2, 4-D (7.5 mgl^{-1}).

Keywords: *Pyrus*, *in vitro*, explant establishment, necrotic cultures, shoot proliferation, aseptic cultures, graft success, vitrification, vigour, explant necrosis and pre-treatment

Signature of Major Advisor **Signature of the Student**

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ਸਾਰ

ਮੌਜੂਦਾ ਅਧਿਐਨ ਪੱਥਰਨਾਖ (*ਪਾਇਰਸ ਪਾਇਰੀਫੋਲੀਆ* (ਬਰਮ ਐਫ.) ਨਕਈ) ਦੀ ਇੰਨ-ਵਿਟਰੋ ਪਰੋਪੇਗੇਸ਼ਨ (ਵਧਣ-ਫੁੱਲਣ) ਅਤੇ ਗ੍ਰਾਫਟਿੰਗ ਦਾ ਮੁਲਾਂਕਣ ਕਰਨ ਲਈ ਪੰਜਾਬ ਖੇਤੀਬਾੜੀ ਯੂਨੀਵਰਸਿਟੀ ਲੁਧਿਆਣਾ ਦੇ ਫ਼ਲ ਵਿਗਿਆਨ ਵਿਭਾਗ ਦੀ ਟਿਸ਼ੂ ਕਲਚਰ ਪ੍ਰਯੋਗਸ਼ਾਲਾ ਵਿਖੇ ਸੰਨ 2011-13 ਦੌਰਾਨ ਕੀਤਾ ਗਿਆ। ਇੰਨ-ਵਿਟਰੋ ਪਰੋਪੇਗੇਸ਼ਨ ਪ੍ਰੋਟੋਕੋਲ ਤਿਆਰ ਕਰਨ ਲਈ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਅਤੇ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਦੇ ਨੋਡਲ ਐਕਸਪਲਾਂਟਾਂ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ। ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਅਤੇ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਦੇ ਸਥਾਪਨ, ਪ੍ਰਸਾਰ ਅਤੇ ਰੂਟਿੰਗ ਉਪਰ ਕਈ ਤਰ੍ਹਾਂ ਦੇ ਮੀਡੀਆ {1/2 ਐਮ.ਐਸ. (ਐਮ.$_1$), ਐਮ.ਐਸ. (ਐਮ.$_2$) ਅਤੇ ਡਬਲਯੂ.ਪੀ.ਐਮ. (ਐਮ.$_3$)} ਅਤੇ ਵਿਕਾਸ ਨਿਰੰਤਰਕਾਂ ਦੇ ਪ੍ਰਭਾਵ ਦਾ ਆਂਕਲਣ ਕੀਤਾ ਗਿਆ। ਵੱਖੋ-ਵੱਖਰੀ ਲੰਬਾਈ ਵਾਲੀਆਂ (ਐਲ$_1$=<5 ਐਮ.ਐਮ., ਐਲ$_1$=5-10 ਐਮ.ਐਮ. ਅਤੇ ਐਲ$_3$=10-15 ਐਮ.ਐਮ.) ਦੋ ਸ਼ੂਟ ਟਿਪਾਂ ਭਾਵ ਇੱਕ ਫੋਰਸਡ ਸ਼ੂਟ-ਟਿੱਪ (ਐਸ$_2$) ਅਤੇ ਦੂਜੀ ਇੰਨ-ਵਿਟਰੋ ਸ਼ੂਟ-ਟਿੱਪ (ਐਸ$_1$) ਤੋਂ ਤਿਆਰ ਕੀਤੀ ਪੱਥਰਨਾਖ ਦੀ ਕਲਮ ਦੀ ਵਰਤੋਂ ਇੰਨ-ਵਟਿਰੋ ਕੈਂਥ ਰੂਟਸਟਾਕ ਉਪਰ ਸ਼ੂਟ-ਟਿੱਪ ਗ੍ਰਾਫਟਿੰਗ ਲਈ ਕੀਤੀ ਗਈ। ਗ੍ਰਾਫਟਿੰਗ ਦੀਆਂ ਦੋ ਵਿਧੀਆਂ ਭਾਵ ਵੈਜ (ਜੀ$_1$) ਅਤੇ ਹੌਰੀਜ਼ੰਟਲ (ਜੀ$_1$) ਦੀ ਵਤਰੋਂ ਕੀਤੀ ਗਈ ਅਤੇ ਇਸ ਤਰ੍ਹਾਂ ਤਿਆਰ ਕੀਤੇ ਗਏ ਸ਼ੂਟ-ਟਿੱਪ ਨੂੰ ਵੱਖੋ-ਵੱਖਰੇ ਮੀਡੀਆ ਭਾਵ ਐਮ.ਐਸ. ਮੀਡੀਅਮ + 20 ਗ੍ਰ/ਲਿ. ਸੂਕਰੋਜ਼ (ਐਮ$_1$), ਐਮ.ਐਸ. ਘੋਲ + 20 ਗ੍ਰ/ਲਿ. ਸੂਕਰੋਜ਼ (ਐਮ$_2$) ਵਾਲੇ ਤਰਲ ਮੀਡੀਅਮ, ਐਮ.ਐਸ. ਮੀਡੀਅਮ + 40 ਗ੍ਰ/ਲਿ. ਸੂਕਰੋਜ਼ (ਐਮ$_3$) ਅਤੇ ਐਮ.ਐਸ. ਘੋਲ + 40 ਗ੍ਰ/ਲਿ. ਸੂਕਰੋਜ਼ (ਐਮ$_4$) ਵਾਲੇ ਤਰਲ ਮੀਡੀਅਮ ਵਿੱਚ ਵਿਕਸਤ ਹੋਣ ਦਿੱਤਾ ਗਿਆ। ਅਸੈਪਟਿਕ ਕਲਚਰ (%), ਪਿਉਂਦ ਦੀ ਸਫਲਤਾ (%), ਨਕਰੋਸਿਸ (%), ਵਰਟੀਫਿਕੇਸ਼ਨ (%) ਅਤੇ ਤਾਕਤ (1-3 ਦਾ ਪੈਮਾਨਾ) ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਇਸ ਤੋਂ ਇਲਾਵਾ, ਗ੍ਰਾਫਟਿੰਗ ਤੋਂ ਪਹਿਲਾਂ ਕਲਮ ਨੂੰ ਟੀ.ਡੀ.ਜ਼ੈਡ. ਅਤੇ 2,4-ਡੀ ਨਾਲ ਇੱਕ ਮਿੰਟ ਤੱਕ ਸੋਧਿਆ ਗਿਆ। ਅਸੈਪਟਿਕ ਕਲਚਰ (%), ਪਿਉਂਦ ਦੀ ਸਫਲਤਾ (%), ਨਕਰੋਸਿਸ (%) ਅਤੇ ਵਰਟੀਫਿਕੇਸ਼ਨ (%) ਅਤੇ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਅਤੇ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਦੋਨਾਂ ਵਿੱਚ ਕਲਮ ਦੇ ਸਥਾਪਨ ਪੜਾਅ ਦੌਰਾਨ ਨੈਕਰੋਟਿਕ ਕਲਚਰ (%) ਉਪਰ ਮੀਡੀਅ ਦੀ ਕਿਸਮ ਅਤੇ ਗ੍ਰੋਥ ਰੈਗੂਲੇਟਰਾਂ ਦਾ ਪ੍ਰਭਾਵ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ। ਬੀ.ਏ.ਪੀ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਅਤੇ ਆਈ.ਬੀ.ਏ. (0.01 ਮਿ.ਗ੍ਰ./ਲਿ.) ਨਾਲ ਫੋਰਟੀਫਾਇਡ ਐਮ$_2$ ਮੀਡੀਅਮ ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਵਿੱਚ ਸਭ ਤੋਂ ਘੱਟ ਨੈਕਰੋਟਿਕ ਕਲਚਰ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਵੇਖਣ ਨੂੰ ਮਿਲੀ ਜਦੋਂਕਿ ਬੀ.ਏ.ਪੀ. (1.5 ਮਿ.ਗ੍ਰ./ਲਿ.) ਅਤੇ ਆਈ.ਬੀ.ਏ. (0.01 ਮਿ.ਗ੍ਰ./ਲਿ.) ਵਾਲੇ ਐਮ$_2$ ਮੀਡੀਅਮ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਲਈ ਸਭ ਤੋਂ ਵਧੇਰੇ ਅਸਰਦਾਰ ਸਿੱਧ ਹੋਇਆ। ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਵਿੱਚ ਬੀ.ਏ.ਪੀ. (1.5 ਮਿ.ਗ੍ਰ./ਲਿ.) ਅਤੇ ਆਈ.ਬੀ.ਏ. (0.01 ਮਿ.ਗ੍ਰ./ਲਿ.) ਨਾਲ ਫੋਰਟੀਫਾਇਡ ਐਮ$_2$ ਮੀਡੀਅਮ ਉਪਰ ਕਲਮ ਦੀ ਸਥਪਨਾ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਸਭ ਤੋਂ ਵਧੇਰੇ (96.10%) ਪਾਈ ਗਈ ਜਦੋਂਕਿ ਪੱਥਰਨਾਖ (ਐਕਟਿਵ) ਵਿੱਚ ਬੀ.ਏ.ਪੀ. (1.5 ਮਿ.ਗ੍ਰ./ਲਿ.) ਅਤੇ ਆਈ.ਬੀ.ਏ. (0.01 ਮਿ.ਗ੍ਰ./ਲਿ.) ਨਾਲ ਫੋਰਟੀਫਾਇਡ ਐਮ$_3$ ਮੀਡੀਅਮ ਉਪਰ ਕਲਮ ਦੀ ਇਸਟੈਬਲਿਸ਼ਮੈਂਟ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਸਭ ਤੋਂ ਵਧੇਰੇ (96.29%) ਪਾਈ ਗਈ। ਇਸੇ ਤਰ੍ਹਾਂ ਬੀ.ਏ.ਪੀ. (1.5 ਮਿ.ਗਾ./ਲਿ.) ਅਤੇ ਆਈ.ਬੀ.ਏ. (0.25 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_2$ ਮੀਡੀਅਮ ਨਾਲ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ 63.60 ਅਤੇ 52.80 ਪ੍ਰਤੀਸ਼ਤ ਐਕਸਪਲਾਂਟ ਦੀ ਸਥਾਪਨਾ ਹੋਈ। ਬੀ.ਏ.ਪੀ. (2.5 ਮਿ.ਗਾ./ਲਿ.) ਅਤੇ ਬੀ.ਏ.ਪੀ. (5.0 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਅਤੇ ਪੱਥਰਨਾਖ (ਐਕਟਿਵ) ਵਿੱਚ ਕਲਚਰ ਦੀ ਪਰੋਲੀਫਿਕੇਸ਼ਨ ਸਭ ਤੋਂ ਵਧੇਰੇ ਕ੍ਰਮਵਾਰ 85.67% ਅਤੇ 79.47% ਹੋਇਆ। ਹਾਲਾਂਕਿ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਬੀ.ਏ.ਪੀ. (3.0 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਸਭ ਤੋਂ ਵਧੇਰੇ ਪਰੋਲੀਫਿਕੇਸ਼ਨ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ। ਇਸੇ ਤਰ੍ਹਾਂ ਬੀ.ਏ.ਪੀ. (2.5 ਮਿ.ਗਾ./ਲਿ.) ਅਤੇ ਬੀ.ਏ.ਪੀ. (3.0 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ

(ਐਕਟਿਵ ਅਤੇ ਫੋਰਸਡ) ਵਿੱਚ ਪ੍ਰਤੀ ਐਕਸਪਲਾਂਟ ਸਭ ਤੋਂ ਵਧੇਰੇ ਸ਼ਾਖਾਵਾਂ ਨਿਕਲੀਆਂ। ਫਿਰ ਵੀ ਬੀ.ਏ.ਪੀ. (2.5 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_2$ ਮੀਡੀਅਮ ਨਾਲ ਪੱਥਰਨਾਖ (ਐਕਟਿਵ) ਵਿੱਚ ਪ੍ਰਤੀ ਐਕਸਪਲਾਂਟ ਸਭ ਤੋਂ ਵਧੇਰੇ ਸ਼ਾਖਾਵਾਂ ਨਿਕਲੀਆਂ। ਕੰਟਰੋਲ ਵਜੋਂ ਵਰਤੇ ਗਏ ਮੀਡੀਅਮ ਭਾਵ ਬੀ.ਏ.ਪੀ. (0.0 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਵਿੱਚ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਦੀਆਂ ਸਭ ਤੋਂ ਲੰਬੀਆਂ ਸ਼ਾਖਾਵਾਂ ਨਿਕਲੀਆਂ। ਬੀ.ਏ.ਪੀ. (0.5 ਮਿ.ਗਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਵਿੱਚ ਸਭ ਤੋਂ ਲੰਬੀਆਂ ਸਾਖਾਵਾਂ (53.75 ਮਿ.ਮੀ.) ਪ੍ਰਾਪਤ ਹੋਈਆਂ। ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਅਤੇ ਕੈਂਥ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਦੋਨਾਂ ਵਿੱਚ ਜੜ੍ਹ-ਮੁੱਢ (%), ਪ੍ਰਤੀ ਐਕਸਪਲਾਂਟ ਮੁੱਢਾਂ ਦੀ ਸੰਖਿਆ ਅਤੇ ਜੜ੍ਹ ਦੀ ਲਬਾਈ ਉਪਰ ਮੀਡੀਅ ਦੀ ਕਿਸਮ ਅਤੇ ਗ੍ਰੋਥ ਰੈਗੂਲੇਟਰਾਂ ਦਾ ਪ੍ਰਭਾਵ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ। ਬੀ.ਏ.ਪੀ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਨਾਲ ਫੋਰਟੀਫਾਇਡ ਐਮ$_1$ ਮੀਡੀਅਮ ਵਿੱਚ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) (10.16%) ਅਤੇ ਪੱਥਰਨਾਖ (ਐਕਟਿਵ) (9.16%) ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਰੂਟਿੰਗ ਹੋਈ ਜਦੋਂਕਿ ਕੈਂਥ (ਫੋਰਸਡ) (13.34%) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) (14.04%) ਵਿੱਚ ਬੀ.ਏ.ਪੀ. (0.1 ਮਿ.ਗ੍ਰ./ਲਿ.) ਵਾਲੇ ਐਮ$_1$ ਮੀਡੀਅਮ ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਰੂਟਿੰਗ ਹੋਈ। ਭਾਵੇਂ ਐਨ.ਏ.ਏ. ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ ਤਾਂ ਕਿਸੇ ਵੀ ਮੀਡੀਆ ਵਿੱਚ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਵਿੱਚ ਕੋਈ ਰੂਟਿੰਗ ਨਹੀ ਹੋਈ ਫਿਰ ਵੀ ਐਨ.ਏ.ਏ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ) ਨੇ ਐਮ$_1$ ਮੀਡੀਅਮ ਉਪਰ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਕ੍ਰਮਵਾਰ 29.61 ਅਤੇ 27.46 ਪ੍ਰਤੀਸ਼ਤ ਰੂਟਿੰਗ ਵਿਖਾਈ। ਆਈ.ਬੀ.ਏ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਵਾਲੇ ਐਮ$_1$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਪੱਥਰਨਾਖ (ਫੋਰਸਡ) ਅਤੇ ਪੱਥਰਨਾਖ (ਐਕਟਿਵ) ਵਿੱਚ ਪ੍ਰਤੀ ਐਕਸਪਲਾਂਟ ਕ੍ਰਮਵਾਰ 2.38 ਅਤੇ 2.60 ਜੜਾਂ ਪਾਈਆਂ ਗਈਆਂ। ਇਸੇ ਤਰ੍ਹਾਂ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਆਈ.ਬੀ.ਏ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਵਾਲੇ ਐਮ$_1$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਸਭ ਤੋਂ ਵਧੇਰੇ ਜੜ੍ਹਾਂ ਪ੍ਰਾਪਤ ਹੋਈਆਂ। ਐਨ.ਏ.ਏ. (1.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਵਾਲੇ ਐਮ$_1$ ਅਤੇ ਐਮ$_2$ ਮੀਡੀਅਮ ਵਿੱਚ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਲੰਬਾਈ ਵਾਲੀਆਂ ਜੜਾਂ (ਕ੍ਰਮਵਾਰ 3.40 ਅਤੇ 3.60) ਦਾ ਵਿਕਾਸ ਕੀਤਾ। ਇਸੇ ਤਰ੍ਹਾਂ ਆਈ.ਬੀ.ਏ. (1.0 ਮਿ.ਗ੍ਰਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਨਾਲ ਪੱਥਰ ਨਾਖ (ਫੋਰਸਡ ਅਤੇ ਐਕਟਿਵ) ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਲੰਬਾਈ ਵਾਲੀਆਂ ਜੜ੍ਹਾਂ ਦਾ ਵਿਕਾਸ ਹੋਇਆ। ਹਾਲਾਂਕਿ ਆਈ.ਬੀ.ਏ. (0.1 ਮਿ.ਗ੍ਰਾ./ਲਿ.) ਵਾਲੇ ਐਮ$_3$ ਮੀਡੀਅਮ ਨਾਲ ਕੈਂਥ (ਫੋਰਸਡ) ਅਤੇ ਕੈਂਥ (ਐਕਟਿਵ) ਵਿੱਚ ਜੜ੍ਹਾਂ ਦੀ ਲੰਬਾਈ ਕ੍ਰਮਵਾਰ 31.15 ਅਤੇ 31.10 ਮਿ.ਮੀ. ਪਾਈ ਗਈ। ਐਨ.ਏ.ਏ. (0.1 ਮਿ.ਗ੍ਰਾ./ਲਿ.) ਨਾਲ ਐਮ$_1$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਕੈਂਥ (ਫੋਰਸਡ) ਵਿੱਚ ਜੜ੍ਹਾਂ ਦੀ ਲੰਭਾਈ ਸਭ ਤੋਂ ਵਧੇਰੇ ਭਾਵ 22.97 ਮਿ.ਮੀ. ਜਦੋਂਕਿ ਐਨ.ਏ.ਏ. (0.1 ਮਿ.ਗ੍ਰਾ./ਲਿ.) ਨਾਲ ਐਮ$_3$ ਮੀਡੀਅਮ ਦੀ ਵਰਤੋਂ ਨਾਲ ਜੜ੍ਹਾਂ ਦੀ ਲੰਭਾਈ ਸਭ ਤੋਂ ਵਧੇਰੇ ਭਾਵ 23.22 ਮਿ.ਮੀ. ਪ੍ਰਾਪਤ ਹੋਈ। ਕਲਮ ਦੇ ਮੂਲ, ਕਲਮ ਦੀ ਲੰਬਾਈ, ਗ੍ਰਾਫਟਿੰਗ ਲਈ ਵਰਤੀ ਜਾਂਦੀ ਵਿਧੀ ਅਤੇ ਸ਼ੂਟ-ਟਿੱਪ ਗ੍ਰਾਫਟਿੰਗ ਦੌਰਾਨ ਵਰਤੇ ਜਾਂਦੇ ਮੀਡੀਅਮ ਦਾ ਪ੍ਰਭਾਵ ਅਸੈਪਟਿਕ ਕਲਚਰ, ਗ੍ਰਾਫਟ ਸਫਲਤਾ, ਨੈਕਰੋਸਿਸ, ਵਿਟਰੀਫਿਕੇਸ਼ਨ ਅਤੇ ਤਾਕਤ ਉਪਰ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ। ਐਸ$_1$, ਐਲ$_2$, ਜੀ$_2$ ਅਤੇ ਐਮ$_1$ ਦੀ ਵਰਤੋਂ ਨਾਲ ਅਸੈਪਟਿਕ ਕਲਚਰ ਸਭ ਤੋਂ ਵਧੇਰੇ ਭਾਵ 84.34% ਸੀ। ਐਸ$_1$, ਐਲ$_2$, ਜੀ$_1$ ਅਤੇ ਐਮ$_2$ ਦੀ ਵਰਤੋਂ ਨਾਲ ਪਿਉਂਦ ਦੀ ਸਫਲਤਾ ਸਭ ਤੋਂ ਵਧੇਰੇ ਭਾਵ 33.71% ਪਾਈ ਗਈ। ਹਾਲਾਂਕਿ ਕਈ ਤਰ੍ਹਾਂ ਦੇ ਉਪਚਾਰਾਂ ਦੀ ਵਿੱਚ ਨੈਕਰੋਸਿਸ ਸਭ ਤੋਂ ਘੱਟ (0.0%) ਸੀ। ਵਿਟ੍ਰੀਫਿਕੇਸ਼ਨ ਉਪਰ ਵੀ ਕਈ ਉਪਚਾਰਾਂ ਦਾ ਪ੍ਰਭਾਵ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ ਅਤੇ ਸਭ ਤੋਂ ਘੱਟ ਵਿਟ੍ਰੀਫਿਕੇਸ਼ਨ ਐਸ$_1$, ਜੀ$_1$, ਐਲ$_1$ ਅਤੇ ਐਮ$_4$ ਵਿੱਚ ਵੇਖਣ ਨੂੰ ਮਿਲੀ। ਐਸ$_1$, ਐਲ$_2$, ਜੀ$_1$ ਅਤੇ ਐਮ$_2$ ਦੀ ਵਰਤੋਂ ਨਾਲ ਪਿਉਂਦ ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਤਾਕਤ ਵੇਖਣ ਨੂੰ ਮਿਲੀ। ਗ੍ਰਾਫਟਿੰਗ ਦੀ ਸਫਲਤਾ ਅਤੇ ਹੋਰ ਮਾਪਦੰਡਾਂ ਉਪਰ ਗ੍ਰੋਥ ਰੈਗੂਲੇਟਰਾਂ ਦੇ ਉਪਚਾਰ ਦਾ ਫਾਇਦੇਮੰਦ ਪ੍ਰਭਾਵ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ। ਟੀ.ਡੀ.ਜ਼ੈਡ. (0.5 ਮਿ.ਗ੍ਰ./ਲਿ.) + 2,4-ਡੀ (5.0 ਮਿ.ਗ੍ਰ./ਲਿ.) ਦੇ ਉਪਚਾਰ ਨਾਲ ਨਾਲ ਸਭ ਤੋਂ ਵਧੇਰੇ ਅਸੈਪਟਿਕ ਕਲਚਰ (88.78%) ਜਦੋਂ ਕਿ ਟੀ.ਡੀ.ਜ਼ੈਡ. (0.5 ਮਿ.ਗ੍ਰ./ਲਿ.) + 2,4-ਡੀ (7.5 ਮਿ.ਗ੍ਰ./ਲਿ.) ਦੇ ਉਪਚਾਰ ਨਾਲ ਸਭ ਤੋਂ ਵਧੇਰੇ ਗ੍ਰਾਫਟਿੰਗ ਸਫਲਤਾ ਮਿਲੀ। ਹਾਲਾਂਕਿ ਟੀ.ਡੀ.ਜ਼ੈਡ. (0.75%) ਦੇ ਉਪਚਾਰ ਨਾਲ ਸਭ ਤੋਂ ਘੱਟ ਨੈਕਰੋਸਿਸ (1.65%) ਅਤੇ ਟੀ.ਡੀ.ਜ਼ੈਡ. (0.75 ਮਿ.ਗ੍ਰ./ਲਿ.) + 2,4-ਡੀ (7.5 ਮਿ.ਗ੍ਰ./ਲਿ.) ਦੇ ਉਪਚਾਰ ਨਾਲ ਸਭ ਤੋਂ ਘੱਟ ਵਿਟ੍ਰੀਫਿਕੇਸ਼ਨ (4.11%) ਵੇਖਣ ਨੂੰ ਮਿਲੀ।

ਮੁੱਖ ਸ਼ਬਦ: *ਪਾਇਰਸ*, ਇੰਨ ਵਿਟਰੋ, ਐਕਸਪਲਾਂਟ ਦੀ ਸਥਾਪਨਾ, ਨੈਕਰੋਟਿਕ ਕਲਚਰ, ਪੈਦਾ ਕਰਨਾ, ਅਸੈਪਟਿਕ ਕਲਚਰ, ਪਿਉਂਦ ਦੀ ਸਫਲਤਾ, ਵਿਟ੍ਰੀਫਿਕੇਸ਼ਨ, ਤਾਕਤ, ਨੈਕਰੋਸਿਸ ਅਤੇ ਉਪਚਾਰ

ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

CONTENT

LIST OF PLATES

LIST OF ABBREVIATIONS

µM	:	Micromolar
2, 4-D	:	2,4 Dichloro Phenoxy Acetic Acid
2ip	:	2-iso-Pentyladenine
½ MS	:	Half strength Murashige and Skoog
BAP	:	Benzyl Amino Purine
cm	:	Centimeter
DKW	:	Driver Kuniyuki Walnut
DTR	:	Difficult to Root
ETR	:	Easy to Root
GA	:	Gibberlic Acid
$HgCl_2$	:	Mercuric Chloride
IAA	:	Indole Acetic Acid
IBA	:	Indole Butyric Acid
LP	:	Lepoivre
mg	:	Milligrams
mgl^{-1}	:	Milligrams per litre
MM	:	Malling Merton
MS	:	Murashige and Skoog
NAA	:	Naphthalene Acetic Acid
NH_4^+	:	Ammonium ion
ppm	:	Parts per million
QL	:	Quorin Lepoivre
TDZ	:	Thiadiazuron
WPM	:	Woody Plant Medium

CHAPTER I

INTRODUCTION

Genus *Pyrus* belongs to the sub-tribe *Pyrinae*, tribe *Pyreae* in the *Spiraeoideae* sub-family of the Rosaceae family (Potter *et al* 2007). This genus is believed to have originated during the Tertiary periods (65–55 million years ago) in the mountainous area of Western and South-Western China and spread East and West from there. Vavilov (1951) identified three centres of diversity for pears i.e. China, Central Asia and Near Eastern/ Asia Minor. The genus *Pyrus* (2n = 34) contains 22 species having basic chromosome numbers of X = 8 and Y = 9 (Bell *et al* 1996). Commercial pear production is mainly represented by *Pyrus communis* (European pear), *Pyrus pyrifolia* (Asian or Oriental pear) and their hybrids. Most of the cultivars belonging to *Pyrus communis* are suitable for cultivation in temperate climate, however, the *Pyrus pyrifolia* cultivars are well adapted to sub-tropical climate of north-western states of India.

Pyrus communis L. is the main species grown in Europe, North America, South America, Africa and Australia. Wild populations of *Pyrus communis* L. var. Pyraster and/or *P. caucasia* Fed. are probable ancestors of cultivated *P. communis* L. and there is some evidence of contribution of *P. nivalis* Jacq. (Challice and Westwood 1973).

The domestic pears of Asia are mostly from *P. pyrifolia* (Burm F.) Nakia, known as Japanese or sand pear. The sand pear, *P. pyrifolia* is the main cultivated species in Southern and Central China and in Japan, but *P. ussuriensis* Maxim, is also grown in North China and Japan (Shen 1980). Hybrids of *P. communis* and *P. pyrifolia* are grown in some parts of North America. Selections of *P. pashia* Buch. Ham. are cultivated in Southern China (Pieniazek 1966) and North India (Mukherjee *et al* 1969).

Worldwide production of European pear cultivars is based on heirloom varieties such as Abbe Fetel, Bartlett (syn. Williams Bon Chretien and its sports), Beurre Anjou, Beurre Bosc (syn. Kai-ser), Conference, Doyenne du Comice, Pack-hams Triumph and Passe Crassane. China accounts for most of the world's Asian pear production with the *P. bretscheneideri* cultivars Dong Shan SuLi, YaLi and Huang HuaLi comprising the largest production area (Gemma 2008). Presently, pear is next to apple in importance, acreage, production and varietal wealth among temperate fruits in India. It can grow under wider temperature conditions ranging from -26°C when dormant to as high as 47°C during growth period (Chadha 2001). Temperate pear can grow from foothills to high hills (600-2700 m amsl) experiencing 500-1500 chilling hours, while the sub-tropical pears require only 200-300 chilling hours.

Total world pear production reached more than 20 million metric tons in 2013 (FAO 2013). Pear is grown from warm humid sub-tropical plains to cold dry temperate regions of

India occupying an area of 49,340 ha with the annual production of 3,17,270 MT (indiastat 2013). The main pear growing areas are located in Jammu and Kashmir, Himachal Pradesh, Punjab, Uttarakhand, Arunachal Pradesh, Manipur, Mizoram, Nagaland and Tamil Nadu. In Punjab, it ranks 4th among fruit crops in terms of area after citrus, guava and mango and occupies an area of 2787 ha with an annual production of 63040 MT (Anonym 2014). The area can be increased further and cultivation of this crop may prove to be a best alternative for diversification of agriculture.

Pear has become an important fruit crop of Punjab particularly due to recommendation of semi-soft pear varieties by Punjab Agricultural University. Current pear production relies on Patharnakh (hard pear) which is the leading cultivar of Punjab followed by Baggugosha and Le Conte. The semi-soft varieties like Punjab Beauty, Punjab Nectar, Punjab Gold and soft varieties like YaLi and Nijjisseiki have been recommended by PAU for general cultivation in the state. Other soft varieties like Shinseiki, Kosui and Hosui are also performing well under local conditions.

Since, the fruiting characteristics of plants produced from seeds are nearly always inferior to the parent plant due to heterozygosity, thus their seed progeny is not true, to the type (Jackson and Looney 1999). Asexual propagation on the other hand gives rise to plants which are genetically identical to the parent plant and thus permits the perpetuation of the unique character of the cultivar.

Tissue culture techniques are becoming increasingly popular as alternative means of plant vegetative propagation. In recent years, tissue culture technique e.g. micro-propagation is increasingly used for rapid clonal multiplication of several economic plants, restoration of vigour and yield lost due to infection and preservation of germplasms. In a relatively short span of time and space, a large number of plants can be produced starting from a single individual.

Seedling rootstocks are not uniform in growth and productivity (Baviera *et al* 1989). Therefore, vegetative propagation methods like cutting and stooling are used to multiply pear rootstocks. In India, the Patharnakh is propagated by rooting of cutting as well as by budding/ grafting on various rootstocks.

In vitro propagation has shown promises for rapid and large scale clonal multiplication of disease-free planting material throughout the year. *In vitro* propagation has been reported in several pear cultivars, *viz.* Seckel (Singha 1982), Conference (Baviera *et al* 1989), Pakham's Truimph and Beurre Bosc (Shen and Mullins 1984), Bartlett (Al Maarri *et al* 1994), Gola (Dwivedi and Bist 1999), Rocha (Freire *et al* 2002), Durondeau (Lucyszyn *et al* 2006) and Sebri (Karimpour *et al* 2013). Similarly, some rootstocks like OH × F 51 (Cheng 1979), OPR 157, OPR 260 and OH × F 230 (Yeo and Reed 1995), *P calleryana* (Antunes de *et al* 2004), *P. communis* L. rootstock (Rahman *et al* 2007), wild pear (Thakur and Kanwar

2008), Pyrodwarf (Ruzic *et al* 2011) and *P. betulaefolia* L. (Hassanen and Gabr 2012) has been propagated *in vitro*. Differences exist among all genotypes for their requirement of basal medium and growth regulators.

The ability to establish shoot tip cultures, proliferate shoots, induce rooting and to acclimatize the resulting plantlets are stages of *in vitro* propagation (Bell and Reed, 2002). The use of tissue culture for fruit and nut tree species have increased substantially since the early 1970s and virtually all temperate fruit tree species have been micropropagated with various degrees of success. Micropropagation protocols have been published for over 20 pear cultivars, including the major *P. communis* L. cultivars, and also several Japanese cultivars of *P. pyrifolia* (Bhojwani *et al* 1984) and *P. calleryana* (Berardi *et al* 1993) as well as for quince (*Cydonia oblonga* L.) rootstock (Dolcet-Sanjuan *et al* 1990).

The necessity to modernize the planting material production technologies of pear has been stimulated by many considerations such as the trends towards increasing the planting densities in field grown trees and the transition to intensive growing systems, which includes the selection of new parents for breeding programmes, development and introduction of new cultivars and the modernization of tree habit and pruning. All of these changes have created a demand for more and more quantities of quality planting material. The conventional system of propagation is not only time consuming but the material raised is neither uniform nor healthy.

Application of *in vitro* techniques in fruit growing can, therefore, be a viable alternative to circumvent these problems. One such application is *in vitro* shoot tip grafting or micrografting. Micrografting was developed in 1980s (Jonard 1986) and consists of the placement of meristem tip or shoots tip explant onto a decapitated rootstock that has been grown aseptically from seed or micropropagation (Hartmann *et al* 2002). The results of *in vitro* micrografting and the plant material derived from it can be further multiplied in tissue culture conditions or acclimatized to out door conditions. Micrografting is a technique that potentially can combine the advantages of rapid *in vitro* multiplication with increased productivity that results from grafting, superior rootstock and scion combinations (Gebhardt and Goldbach 1988). The juvenile phase can be circumvented by micrografting on to a rootstock that induces early maturity. Besides to the benefits of traditional grafting, micrografting shoot tips can be an efficient means of regenerating plant material free of endogenous microbial contaminants (Zilka *et al* 2002) with enhanced potential for true-to-type cloning mature plants (Franclet 1983). Micrografting technique appears to be useful in early screening of graft compatibility in pear as the overlapping of different stress response induced by the graft itself at the interphase between the partners makes identification of the mechanism underlying localized incompatibility difficult (Pirovano *et al* 2002). Micrografting has application for physiological analysis of the rejuvenation of mature phase plants (Jonard

1986). It is possible to carry shoot-tip grafting at any time of the year (Musacchi *et al* 2004) since grafting is done under *in vitro* conditions.

Grafting under *in vitro* conditions has several advantages both for production and research. *In vitro* shoot-tip grafting has often been applied for the (i) improvement and rejuvenation of several tree species (Canon *et al* 2006, Fabiana *et al* 2006) (ii) virus elimination (Bisognin *et al* 2008, Ribeiro *et al* 2008) (iii) study of physiological connections between rootstocks and scions such as (in)compatibility, root to shoot communication or transport (Nelson 2004, Bortolotti *et al* 2005) and (iv) use in quarantine as this method has a minimum risk for importing plants (George 1993).

Due to the multiple uses and advantages of shoot tip grafting, this technology would be of interest or of potential practical value to technicians, researchers and nursery operators. Professionals in each of these areas would benefit from the introduction of a simpler and more efficient micrografting procedure that is less dependent on mastery of complex techniques and thus will contribute to the practical utility of micrografting as a tool in fruit tree biotechnology.

Keeping in view the popularity and increased demand of Patharnakh pear by fruit growers in Punjab, there is a need to provide high quality true-to-type plants of this cultivar on Kainth rootstock for distribution among fruit growers.

Thus the present study was undertaken with the following objectives:

Objectives

i) To standardize the efficient micropropagation protocols of pear cv. Patharnakh and Kainth rootstock.

ii) To standardize technique of shoot-tip grafting in pear cv. Patharnakh.

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CHAPTER II

REVIEW OF LITERATURE

Plant tissue culture is the back bone of plant biotechnology i.e micropropagation, induction of somaclonal variation, somatic hybridization, cryopreservation, genetic transformation etc. Plant cell and tissue culture has contributed significantly to crop improvement and has potential for the future. Plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cell, tissue and organ under sterile and controlled environmental conditions. Tissue culture techniques are now being widely applied for improvement of horticulture and plantation crops. This technology is being exploited mainly for large scale production and micropropagation of elite planting material with desirable characteristics. This technology has been commercialized globally and has contributed significantly towards the enhanced and mass production of high quality true-to-type planting material. The use of tissue culture for fruit and nut species has increased substantially since the early 1970's with various degree of success.

Shoot tip grafting/Micrografting developed in 1980s (Jonard, 1986) consists of the placement of a maintained scion onto an *in vitro* grown rootstock under aseptic environment. The results of *in vitro* micrografting and the plant material derived from it can be further multiplied in tissue culture conditions or acclimatized to out door conditions. Micrografting is a technique that potentially can combine the advantages of mass and rapid *in vitro* multiplication of disease free planting material with increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach, 1988).

The literature relevant to *in vitro* propagation/ micropropagation and shoot tip grafting of pear is reviewed under the following headings.

2.1 Nutrient media

2.2 *In vitro* propagation/micropropagation

2.2.1 Explant source

2.2.2 Explant size

2.2.3 Explant sterilization

2.3 Growth regulators

2.3.1 Culture establishment

2.3.2 Shoot proliferation

2.3.3 Rooting

2.4 Shoot tip grafting/ Micrografting

2.4.1 Scion origin

2.4.2 Scion length

2.4.3 Media

2.4.4 Graft method

2.4.5 Pre-treatment of growth regulators

2.4.6 Vitrification

2.1 Nutrient media

The concept of growing plants from individual cells was suggested by Haberlandt (1902), who tried to grow leaf cell cultures in simple mineral solutions. Various modifications in nutrient media has been reported by Tukey (1933), White (1943), Nitsch (1951), Heller (1953), Gautheret (1957), Murashige and Skoog (1962), Gamborg *et al* (1976), Favre (1977), Cheng (1979) and Lloyd and McCown (1980). Virtually all media contain inorganic salts and sucrose as a carbon source. Concentration of these ingredients in basal medium depends on the type of plant being cultured and the stage of culture development (Murashige 1974). The pH of the nutrient medium is also a critical factor; most media have pH values between 5.5 and 6.2.

In most cases, Murashige and Skoog (MS) revised medium (1962) was used as mineral medium for culturing many *Pyrus* species and cultivars for regeneration and/or proliferation, sub-culturing and subsequent rooting (Bell and Reed 2002). The basal MS medium contains a relatively high level of nitrogen in the form of ammonium and nitrate ions in addition to high concentration of calcium than other media. The most commonly included vitamins are thiamine, nicotinic acid and pyridoxine. Thiamine is critical and is usually provided in the range of 0.1 to 0.4 mgl^{-1}. Inositol is not essential; nevertheless, it has been clearly beneficial and has been used at the rate of 100 mgl^{-1} (Murashige 1974).

A number of carbohydrates have been used in fruit tissue culture media, but the sucrose has been the most popular and versatile. It is generally incorporated at a concentration of 2-3 per cent. Carbohydrates serve two principle functions (a) they provide an energy source to the tissues, and (b) they maintain an osmotic balance with the medium (Skirvin 1983). Carbohydrates effect the growth and the frequencies of shoot developed and shoot proliferation of woody Rosaceous species (Marino *et al* 1993). Sorbitol produced good results for shoot multiplication of *P. pyrifolia* as compared to sucrose and fructose (Kadota *et al* 2001). Thakur (2004) reported that in various pear species, woody plant medium resulted in the highest shoot proliferation, however, it was at par with Murashige and Skoog medium.

One of the most common organic complexes used in tissue culture is agar. The MS medium includes 10.00 g/l (1%) agar, but many researchers have reduced agar concentration to 8.00g/l or even less. The osmotic potential of high agar concentration to 8.00 g/l or even less. The osmotic potential of high agar concentration and the concomitant reduction in nutrient and organic matter availability to the tissue is the common reason for reducing the agar concentration (Skirvin 1983). In *P. communis* cv. Seckel increasing agar concentrations resulted in decreased shoot growth, but shoot proliferation was significantly increased at

concentrations of 0.6 per cent and higher as compared to 0.3 per cent or lower agar concentrations (Singha 1982). Kadota *et al* (2001) recommended that low concentrations of agar (0.4 and 0.6 %) must be avoided for proliferating *P. pyrifolia* as it increased vitrification at low concentrations. However, 0.8 per cent agar had a better effect than 1.0 and 1.2 per cent agar for shoot proliferation.

Leite *et al* (1997) studied the effect of gelling agents on proliferation and growth of pear shoots. The best shoot proliferation and growth were obtained with explants cultured on medium containing Cialgas agar at any one of the concentration (0.6 or 1.2%) and with Difco agar at 0.6 or 0.8 per cent. Gelrite induced vitrification. Zimmerman *et al* (1995) advocated the use of a mixture of maize starch and gelrite as a gelling agent for micropropgation of *P. communis* cvs Anjou and Seckel supplemented with polysaccharide hydric control agent to eliminate hyperhydricity. The resulting shoot proliferation with Starch Gelrite mixture equaled or exceeded that on the agar gelled medium. Moreever, the Starch-Gelrite mixture is easy to prepare and costs are only 10-15 per cent of agar, or less if starch is purchased in bulk. Although the opaque- grey-white medium makes it more difficult to detect internal contaminants, external contaminants were easily discerned.

Lucyszyn *et al* (2006) used modified gelled medium for micropropagation of Durondeau pear. The production of multiple shoots and formation of roots from shoots were compared with the control solidified with agar alone at a concentration of 0.6 % (w/v). In the media solidified with the mixtures of agar/guar and agar/acarria gelled media, an increase of 32 and 17 %, respectively, was obtained in the number of regenerated shoots. The modified media promoted a higher number of roots and increased the rooting percentage. A maximum of 91 % rooting was obtained in the medium solidified with the agar/acarria gelled media and containing 9.80 µM indole-3-butyric acid. Less callus formation at the base of the shoot was also observed on this medium.

Many researchers have completely eliminated the use of gelling agent for their culture by using shaking or roller drum culture and/ or by using the so called Heller (1949) bridges made of filter papers and supported in liquid medium. Lane (1979c) reported that the cultures of *Prunus cistena* did not root well when agar was present in the medium, so the cultures were rooted in liquid medium using Heller bridges. The cause for this inhibition was unknown, but Kohlenbach and Wernicke (1978) have reported that agar can be toxic to certain plant cultures.

Murashige and Skoog medium has been used for the micropropagation of *P. pyrifolia* (Bhojwani *et al* 1984); *P. communis* L. cvs. Williams, Pakham's Triumph and Beurre Bosc (Shen and Mullins 1984); *P. communis* L. cv. Conference (Baviera *et al* 1989); *P. calleryana* (Berardi *et al* 1993); *P. bretschneideri* cv. Jinhua (Wang *et al* 1994); *P. syrica* (Shibli *et al* 1997): compact clones of *P. communis* L. (Predieri and Govoni 1998). *P. calleryana* (Pasqual

et al 2002a); *P. betulaefolia* (Hassanen and Gabr 2012, Pasqual *et al* 2002b); *P. communis* L. cv. William's (Grigoriadou *et al* 2000), *P communis* L. cv. IGE 2002 (selection of Dr. Jules Guyot) (Iglesias *et al* 2004), *P. pyrifolia* cv. Patharnakh (Thakur and Kanwar, 2008a), *P. serotina* (Thakur and Kanwar, 2008b), *P. communis* L. cv. Sebri (Karimpour *et al* 2013).

Although in pear, most published micropropagation methods used Murashige and Skoog (MS) basal medium yet, some other media with slight modifications have also been used. Lepoivre (LP), Driver-Kuniyuki Walnut (DKW) and Woody Plant Media (WPM) have improved shoot proliferation rates (Bell and Reed 2002, Bell *et al* 2009). These modified media differ from MS in nitrogen concentration or source and calcium concentration.

Al Maarri *et al* (1986) found Lepoivre medium (Quoirin *et al* 1977) to be more efficient than MS medium for micropropagtion of Passe-Crassane pear, in contrast to results from Baviera *et al* (1989) on Conference pear. Likewise, Nedelcheva (1986) compared five basal media (MS, Lepoivre, Schenk and Hildebrandt, Gamborg and White) and found that the greatest number of rapidly growing buds of the cultivar William's was produced on Lepoivre medium. The main features of Lepoivre medium are its low content of NH_4^+ (4.9 mM) and relatively high Ca^{++} content (5.08 mM).

QL medium (Quoirin and Lepoivre 1977) was the best for shoot proliferation of highland pear (Grigoriadou *et al* 2000). Freire *et al* (2002) reported the superiority of QL medium for the establishment of *P. communis* L. cv. Rocha uninodal cuttings.

Yeo and Reed (1995) evaluated Cheng and WPM containing a range of BA, NAA and IBA concentrations as potential medium for shoot multiplication of three *Pyrus* rootstocks OPR 157, OPR 260 and OH × F230. Cheng's medium with 8 µM BA was the best for multiplication of all the genotypes, but auxin types varied. The lower concentration of major elements in WPM (Lloyd and McCown 1980) was more suitable for micropropagation of OH × F (34, 51, 69, 87 and 230) pear rootstocks than the higher concentration in MS medium (Nadosy 1997). Similarly, WPM was more efficient than MS in inducing shoot proliferation in *P. pashia* (Dwivedi and Bist 1997) and *P. pyrifolia* cv. Gola (Dwivedi and Bist 1999). Kadota *et al* (2001) found that though WPM produced highest number of shoots per explant in Japanese pear, yet it was at par with full-strength and half-strength MS medium. Viseur (1987) recommended the use of double phase medium for micropropagation of *P. communis* L. cvs. Durondeau, Conference, Doyenne' du Comice and Professeur Molon base on the mineral composition which is intermediate between Lepoivre and MS media. Double-phase medium base on WPM produced higher number of axillary shoots than ½ MS in Japanese pear (Kadota *et al* 2001).

2.2 *In vitro* propagation/ micropropagation

In pear, micropropagation was achieved for the first time in 1979 on pear rootstock OH × F51 (Cheng 1979) and scion variety Bartlett (Lane 1979b). Micropropagation is the

rapid asexual multiplication *in vitro* of a desired plant (Zimmerman 1983). Most commonly micropropagation is performed using a meristem tip, shoot tip or bud which is induced to grow and then to proliferate in culture. Once a sufficient number of shoots have been produced, they are rooted to yield the plants. This technique uses only differentiated tissue. However, some alternative methods start with differentiated tissue (leaves, petioles or stem) but callus is produced from it. Using this callus, one approach is to induce the formation of adventitious buds which can be grown into shoots and then rooted.

2.2.1 Explant source

Explant source, its maturity, type, season of collection all affect the success or failure of development of micropropagation protocol. Shoot tips from current season's growth have been widely used as explants for *in vitro* propagation of pear. Besides shoot tip which is just one per shoot, nodal buds have also been tried by various workers with good success. Thakur and Kanwar (2008b) observed higher explant establishment with nodal segments as compared to shoot tips in various cultivars of pear. The success of micropropagation also depends on the season, its conditions during which explants are collected. Higher explant establishment and lower contamination and explant browning frequencies were recorded when the explants were obtained during spring and winter season as compared rainy season in Patharnakh, Shaira and Punjab Beauty pear (Thakur, 2004).

Shoots of Sebri cultivar grown in *in vivo* conditions were used as a source of plant material for micropropagation (Karimpour *et al* 2013). Current season shoots were taken during late winter from 20-year-old trees. Hassanen and Gabr (2012) developed an effective *in vitro* culture system from stem segments and shoot tips of field grown pear (*Pyrus betulaefolia*). Peer *et al* (2013) used forced shoot tip explants developed under controlled conditions in the growth chamber obtained from dormant cutting collected from field grown stock plants for culture establishment in sweet cherry cv. Bigarreau Noir Grossa.

2.2.2 Explant size

Explants ranging from 0.2 to 20 mm have been used for micropropagation of pear. Zhao (1982) cultured 0.5 mm shoot tips of *P. pyrifolia* cvs. Jinfeng and Zaosu for their *in vitro* propagation. Nicolodi and Pieber (1989) used 0.2-0.3 mm long meristems with upto three leaf primodia for the micropropagation of *P. betulaefolia*. Banno *et al* (1989) used shoot tips (<0.5 mm) for micropropagation of six Asian pear cultivars. Shibli *et al* (1997) used shoot tips ranging from 0.5-0.7 mm in *P. syrica*. Meristem tips (0.5-1.5 mm) were used for micropropagation of four cultivars of *P. communis* L. Bhojwani *et al* (1984) used 1.0 cm shoot tips in *P. pyrifolia*. Wang *et al* (1994) used 10-15 mm nodal segments for micropropagation of *P. bretschneideri*. Ten mm long nodal segments were used by Shen and Mullins (1984) in *P. communis*. Similarly, 10-15 mm long shoot tips were used by Dwivedi and Bist (1997, 1999) for *in vitro* clonal multiplication of *P. pashia* and *P. pyrifolia* cv. Gola.

Predieri and Govoni (1998) used 15-20 mm shoot tips for the micropropagation of compact clones of *P. communis* L. Twenty mm long shoot tips were used as explants for *in vitro* shoot proliferation of *P. communis* L. cv. Seckel (Singha 1982).

Yeo and Reed (1995) cultured single node explants for the micropropagation of three *Pyrus* rootstocks. Similarly, uninodal explants were used in *P. communis* cv. Rocha (Freire *et al* 2002). The size of the explant determines the survival of the culture. In general the larger the explant size, the better the chance of survival (Thakur 2004). Hence, large explants such as shoot tips and buds should be selected for *in vitro* micropropagation instead of the minute meristems. However, when eradication of viral infection is one of the objectives, meristems of the smallest size should be used (Hu and wang 1983).

2.2.3 Explant sterilization

Plant parts carry a wide range of contaminants and hence obtaining sterile plant material is very difficult. The woody plants are grown in soil for many years under ambient conditions and they are routinely infected with microorganisms both internally and externally which are often difficult to control *in vitro* (Skirvin 1983). Therefore, explants need surface sterilization before culturing. This is done by using various surface sterilizing agents. The kind, concentration and duration of disinfection treatment depend upon the degree of contamination and the hardiness of explant. Many sterilizing agents such as calcium hypochlorite, chlorine water, bleaching water, mercuric chloride, hydrogen peroxide etc have been used. Keeping the shoot tips and nodal segments in running water for an hour prior to a single surface sterilization has been found effective. This treatment also caused leaching of water soluble phenols and other growth inhibitors (Jones *et al* 1978) and effectively reduced the infection (Hughes 1981).

Lane (1979b) surface sterlizd Bartlett pear shoot tips with 2.5 per cent sodium hypochlorite for 15 minutes. Singha (1980) used 0.52 per cent sodium hypochlorite for 10 minutes for glasshouse grown 'Seckel' pear. Bhojwani *et al* (1984) sterilized the explants of *P. pyrifolia* with 0.6 per cent sodium hypochlorite for 30 minutes. They found that shoot tips or buds from field grown trees were more difficult to disinfect in comparison to glasshouse grown cultivars. In *P. communis* L., Viseur (1987) sterilized the explants with 94 per cent ethanol for 5 minutes, followed by 9 per cent calcium hypochlorite for 20 minutes, while, Baviera *et al* (1989) used 0.7 per cent sodium hypochlorite solution in active chlorine for 20 minutes for sterilizing *P. communis* L. cv. Conference.

Further, infectious contaminants have been reported to be removed by placing the Japanese pear explants in 70 per cent alcohol for a few seconds, followed by 5-25 minutes dip in 1-10 per cent sodium hypochlorite containing a few drops of Tween 20 (Banno *et al* 1989, 1992). Similarly, Berardi *et al* (1993) surface sterilized the seeds of open-pollinated *P. calleryana* by dipping, first in 70 per cent ethanol for few seconds, then in sodium hypochlorite

solution (0.18%) chlorine) for 25-30 minutes. Al Maarri *et al* (1994) sterilized explants of *P. communis* L. by dipping them in 95 per cent ethanol for 30 seconds, then immersing them for 20 minutes in 10 per cent Domstos (a commercial preparation of sodium hypochlorite, with 7 per cent active chlorine). In *P. pashia* (Dwivedi and Bist 1997) and in *P. pyrifolia* cv. Gola best surface sterilization was achieved by sequentially dipping the explants in 0.1 per cent $HgCl_2$ for 4 minutes followed by 10 per cent NaOCl for 10 minutes (Dwivedi and Bist 1999). Peer *et al* (2013) obtained maximum aseptic cultures and explant survival in sweet cherry cv. Bigarreau Noir Grossa by using 0.1 $HgCl_2$ for 10 minutes as sterilisation agent.

Internal contamination also posed serious problems in woody plants and this problem has been overcome by adding 10 mgl^{-1} benomyl or benlate to the culture medium or by treating the tissues with these fungicides before disinfection with commercial bleach as normal; disinfection techniques failed to remove the internal contamination (Torres 1988).

2.3 Growth regulators

A balance between endogenous and exogenous growth regulators controls the initiation and development of shoots, roots, plantlet and callus. Skoog and Miller (1957) reported for the first time that the ratio of auxin to cytokinin determines the type and extent of organogenesis in tissue cultures. In general, cytokinins favour meristem proliferation, auxins induce callus formation and rooting and gibberlins induce stem elongation (Kumar and Kumar 1998).

Sedlak and Paprstein (2003) studied the effect of different concentration of the plant growth regulators BAP (6-benzylaminopurine), IBA (indole-butyric acid) and TDZ (thidiazuron) on *in vitro* performance of pear (*Pyrus communis* L.) cv. Koporecka. Micropropagation was improved by culture on MS medium. The highest proliferation rate 5.6 was obtained on medium containing 2 mgl^{-1} BAP, but this BAP concentration caused shorter shoots (0-0.5 cm) with growth abnormalities including 86.7 per cent of these shoots that had narrow and non-expanded leaves. The shoots failed to proliferate and died after rooting phase and subsequent transfer to non-sterile conditions. The combination of 1 mgl^{-1} BAP with 0.1 mgl^{-1} IBA for multiplication produced 2.5 new shoots with larger and well-expanded leaves, which were ideal for acclimatization. A concentration of 1.0 mgl^{-1} TDZ caused abundant callus at the explant base and high level of hyperhydricity in growing shoots. Microcuttings, coming from each proliferation medium, were rooted in MS medium with 1.0 mgl^{-1} IBA. The micropropagated plants with good foliage showed 79.2 per cent survival after rooting and transfer to soil.

2.3.1 Culture establishment

It is considered as first stage in development of protocol for micropropagation of any species. In this stage explants after sterilization are cultured on autoclaved media for their establishment. Either basal media or modified media supplemented with various growth

regulators are used during establishment stage of micropropagation. Explant source, its stage, season, growing conditions, media composition etc. affect the explant establishment. Browning is caused by exudation of phenolic compounds from cut ends of explants into the medium. These compounds can be found in cytoplasm, vacuoles and cell walls. No tissue lacks phenolic compounds and high concentration can be found in actively growing cells (Esau 1977) which modulate plant development (Arnaldos *et al* 2001).

Tissues are injured during explant preparation. This often causes the release of various phenolic compounds that are air oxidized (Robinson 1983), oxidized by peroxidases (Loomis and Battaile 1966; Vaugh and Duke 1984) or polyphenol oxidase and change the metabolism of tissue cells and form brown quinine substances (Chen and Gong, 2005, Leng *et al* 2009). These brown quinine substances gradually enter the tissues and repress the activity of enzymes, resulting in lethal darkening of both tissues and culture medium. The build up of toxic brown or black substances on wounded surface of cultured tissues was listed as one of the basic problems of micropropagation by Pierik (1988).

Explant establishment is a key step in the production of plantlets through tissue culture, but is severely retarded by browning phenomenon. In this context browning assumes great importance and deserves careful attention. Similarly, the first difficulty encountered in the micrografting of fruit cultivars is the oxidation of phenolic compounds at the cut surfaces of the dissected part of apex and stock.

Hassanen and Gabr (2012) reported that 90 per cent of stem segments and 20.50 per cent of shoot tips explant of pear (*Pyrus betulaefolia*) remained aseptic after two weeks of inoculation on establishment medium. The highest significant survival percentage and maximum shoot length of shoot tips was obtained on MS medium containing 1.5 mgl^{-1} BAP and 0.05 mgl^{-1} NAA, while as highest survival percentage and maximum shoot length of nodal segments (stem segments) was obtained on MS medium supplemented with 2.0 mgl^{-1} BAP and 0.05 mgl^{-1} NAA.

Karimpour *et al* (2013) reported the percentage of establishment and callus production were significantly affected by IBA concentration. With no IBA and with a lower IBA concentration (0.1 mgl^{-1}) samples led to more successful establishments of culture (73.57 and 82.2 per cent, respectively) while high IBA concentrations (0.3 and 0.5 mgl^{-1}) lead to the most callus induction (32.6 and 37.6 per cent, respectively). The percentage of browned explants was not affected by IBA concentration. Increasing IBA concentration caused decrease in bud establishment percentage. Maximum bud establishment was obtained at 0 and 0.1 mgl^{-1}, whilst the lowest being resulted at 0.3 and 0.5 mgl^{-1}.

Peer *et al* (2013) reported maximum establishment (50.57 %) of sweet cherry cv. Bigarreau Noir Grossa on MS medium supplemented with BAP (0.25 mgl^{-1}) and kinetin (0.25 mgl^{-1}) and this treatment combination resulted in resulted in maximum proliferating cultures

(99.96 %) with highest multiplication efficiency in terms of proliferation grade (4.0) and shoots/explant (17.28).

Dalal *et al* (2004) achieved an efficient *in vitro* initiating culture system of forced primary explants of M-7 and M-9 apple rootstock. The explants were inoculated in Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine at 0.5 mgl^{-1} and indole-3-butyric acid at 0.1 mgl^{-1} and immediately transferred to low temperature regime (4±3°C) under complete darkness for 2 days followed by subculture on fresh medium and incubating for 5±1 weeks at 24±1°C under 16/8 hour photoperiod. This resulted in considerable reduction of explant browning and phenolic exudation accompanied by enhancement in explant survival.

Kermani *et al* (2008) reported inhibition of production of phenolic compounds in apple rootstocks MM-106 and MM-111 by using a combination of ascorbic acid and citric acid. This resulted in the best initiation medium when the explants were kept at 4°C in dark for six days initially and then transferred to a culture room at 21±1°C temperature with 16/8 hour photoperiod.

Ciccotti *et al* (2008) developed an efficient micropropagation protocol for *in vitro* establishment, multiplication and rooting of the apple proliferation resistant apomictic (*Malus sieboldii*) genotypes. Satisfying results were obtained with the studied genotypes when actively growing shoots were used and explants were soaked in tap water for at least one hour before sterilization. Use of ascorbic acid helped to reduce oxidation in the establishment stage.

2.3.2 Shoot proliferation

The main objective of this stage is to produce maximum number of axillary shoots. In "axillary shoot proliferation", cytokinins are utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. The effective concentration of exogenous cytokinin required to reverse apical dominance varies with the culture systems. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by, in decreasing order, kinetin and 2-iso-pentyladenine (2ip) (Hu and Wang 1983).

Marino (1984) got the proliferation rate of 10-12:1 when shoots of pear cv. Williams Bon Chretion without their tips were cultured on a modified MS medium with 3 per cent sucrose, 0.7 per cent agar and 1.0-1.5 mgl^{-1} BA with or without 0.1 mgl^{-1} IBA or NAA. Cosac and Frasin (2008) established a protocol for regeneration of ten pear cultivars wherein the highest multiplication rate was obtained on MS medium supplemented with 0.1 mgl^{-1} BAP. Gulsen and Dumanoglu (1991) studied the effect of sucrose, agar and pH on shoot multiplication and quality in quince micropropagation. The best shoot proliferation and development was obtained from 30 g/l sucrose, 5 g/l agar and 5.5 pH.

BAP is the most frequently used cytokinin for pear micropropagation, at concentration ranging from 4.4 to 22.2 µM (Chevreau *et al* 1992). Lane (1979b) reported 10

µM BAP as the best concentration for shoot proliferation in *P. communis* L. but the shoots failed to elongate and were often fascinated, whereas, 5 µM BAP was found to be effective for healthy shoot proliferation and produced shoots which could be used readily for root initiation. Lone application of 1 µM BAP has been effective in shoot multiplication; however, addition of 0.3 µM GA_3 and 2.5 µM NAA with BAP was effective for shoot elongation in *P. communis* cv. Seckel (Singha 1980).

Zhao (1982) successfully proliferated Japanese pear cvs. Jinfeng and Zaosu, shoot tips pre-treated with GA_3 (50-200 mgl^{-1}) on MS medium supplemented with 1 mgl^{-1}+ 10 mgl^{-1} GA_3. Growth was better in a Jinfeng as compared to Zaosu.

Shen and Mullins (1984) studied different combinations of cytokinins and found that the association of BAP (6 to 10 µM) with Zeatin (4.5 µM) and 2-iP (4.9 µM) was optimum for the proliferation of the *P. communis* L. cultivars Williams, Pakham's Triumph and Beurre Bosc. The optimum concentration of BAP varied with the cultivar (William's, 6 µM; Pakham's Triumph, 8 µM; Beurre Bosc, 10 µM). In all cultivars, 4-6 axillary shoots per explant were obtained in 6 sub-cultures. In *P. pyrifolia* a large stock of shoots was produced up by multiplication on MS + 1.5 mgl^{-1} BAP + 0.02 mgl^{-1} NAA at a rate of 2.5-fold increase every 6 weeks (Bhojwani *et al* 1984).

Banno *et al* (1989) recommended 1.0 mgl^{-1} BA and 0.1-0.5 mgl^{-1} IBA for the optimum shoot growth and proliferation of Japanese pear cultivars Kosui, Hosui, Osa-Nijisseiki, Yagumo, Shinsui and Nijisseiki. Japanese pear rootstock explants proliferated best in the medium supplemented with 1.0 mgl^{-1} and 0.1 mgl^{-1} IBA (Banno *et al* 1988).

Nicolodi and Pieber (1989) micropropagated three clones of *P. betulaefolia*. Two clones 5 and 9 showed maximum shoot multiplication on the medium supplemented with BAP (2 mgl^{-1}) and NAA (0.1 mgl^{-1}), while clone 3 required a higher concentration of BA (4.0 mgl^{-1} with NAA 0.1 mgl^{-1}).

Stimart and Harbage (1989) observed that shoot proliferation in *P. calleryana* increased as BA level increased but it decreased with increase in concentration of IBA. Highest shoot proliferation was on medium in which IBA was excluded. Shoots were longest on medium containing 1.0 or 2.0 µM BA. Medium with 2.0 µM BA and 0.5 µM IBA was optimum for shoot proliferation and length.

Berardi *et al* (1993) reported no shoot proliferation in *P. calleryana* in the absence of cytokinin. The highest proliferation rate was attained at 1 mgl^{-1} BAP, while the longest shoots were at 0.5 mgl^{-1} BAP. NAA had no influence on shoot proliferation and length. Liaw *et al* (1992) obtained 5.8 and 9.7 shoots of *P. serotina* cv. Hengshan and *P. kawakamii* cv. Niauli respectively within 30 days of transfer of established explants to MS medium containing BA (2.0 mgl^{-1}), kinetin (0.5 mgl^{-1}) and adenine sulphate (4.0 mgl^{-1}).

Baviera *et al* (1989) reported positive correlation between BAP concentration and shoot multiplication rate in *P. communis* L. cv. Conference with BAP (1.0 mgl^{-1}) and NAA (0.01 mgl^{-1}) as the best combination for its shoot multiplication.

Yeo and Reed (1995), while working on micropropagation of three rootstocks, OPR 157, OPR 260 and OH × F 230 reported that 8.0 µM BA and 0.5 µM IBA was the best growth regulator combination for shoot proliferation of OPR 260 and OH × F 230. Shoot proliferation of OPR 157 was the best on 8.0 µM BA, and better on low NAA (0.5 µM) or no auxin than on IBA. Nadosy (1997) developed micropropagation techniques for American-bred hybrid pear rootstocks (OH × F 34, 51, 69, 87, 230), BA 29, pear seedlings and cultivars (Clapp's Favorite and Bartlett). Low concentration of auxins (0.5 mgl^{-1} IAA) and BA (0.5mgl^{-1}) were more effective for shoot proliferation than higher concentrations.

Shibli *et al* (1997) reported enhanced shoot proliferation of wild pear (*P. syrica*) at 1.5 and 2.0 mgl^{-1}. Shoots were compact at higher concentrations of BA and leaves were smaller. Lower BA concentrations and the control induced lower shoot counts and longer internode length. BA was more effective in inducing shoot proliferation than zeatin. The highest number of shoots per explant were obtained with BA at 1.5 mgl^{-1}.

In *P. pashia*, Dwivedi and Bist (1997) found that the number of shoots per culture increased with increase in BA level upto 2 mgl^{-1}. Further, increase in the levels of BA reduced the number of shoots per culture. The combination of 2 mgl^{-1} BA with 0.5 or 1.0 mgl^{-1} IBA resulted in highest number of shoots (7.0) per culture. Liete *et al* (1997) studied the effect of BAP and NAA concentration on *in vitro* multiplication of pear cv. Bartlett and clone OH × F 97. The number of shoots per explant was highest (3.87) in Bartlett with 2.4 mgl^{-1} BA. Neither BA nor NAA had any effect on number of shoots per explant in OH × F 97. NAA at 0.16 mgl^{-1} significantly increased shoot length in Bartlett explants. Increasing BA concentration decreased shoot length in both explants.

Dwivedi and Bist (1999) reported an increase in the number of shoots per explant upto 1.5 mgl^{-1} BA in *P. pyrifolia* cv. Gola. BA and IBA interacted significantly in increasing the number of shoots per culture. The combination of BA (1.5 mgl^{-1}) and IBA (1.0 mgl^{-1}) lead to maximum shoot multiplication (4.32).

Thakur (2004) reported that BA (2.0 mgl^{-1}) + IBA (0.5 mgl^{-1}) induced maximum shoot proliferation in wild pear, Kainth and Shiara whereas, in Punjab Beauty and Patharnakh, the highest shoot proliferation rates were obtained with 1.5 mgl^{-1} BA + 0.5 mgl^{-1} IBA.

Karimpour *et al* (2013) reported that in pear cv. Sebri the highest number of proliferated shoots (2.6-3.6) can be obtained at 3 mgl^{-1} BAP concentration while as higher BAP concentrations led to decline in shoot length.

Hassanen and Gabr (2012) reported that MS medium supplemented with BAP (2.5 mgl^{-1}) and 2iP (2.0 mgl^{-1}) induced the highest number of shoots (8.90), while as BAP (2.0 mgl^{-1}) and 2iP (1.5 mgl^{-1}) resulted in maximum shoot length (4.7 cm) in *P. betulaefolia*.

Ruzic *et al* (2011) reported the highest multiplication index (2.89) of Pyrodwarf pear on MS medium supplemented with 5.0 µM BA and 0.5 µM IBA followed by multiplication index (2.72) on medium fortified with 10.0 µM BA and 2.5 µM IBA. Maximum length of axial shoot (2.62 cm) was obtained on medium supplemented with 5.0 µM BA, while the maximum lateral shoot length (1.47 cm) was obtained on MS medium supplemented with 5.0 µM BA and 2.5 µM IBA.

Prdieri and Govoni (1998) micropropagated seven compact pear clones obtained through *in vitro* irradiation and two standard growth cultivars 'Conference' and Doyenne d' Hiver'. All compact clones, except clone F8, had the highest proliferation rates with the highest tested concentration of BAP (17.6 µM). This contrasted with standard cultivar response. Conference had the highest shoot proliferation with 4.4 or 8.8 µM BA and Doyenne'd'Hiver with 4.4 µM BAP. Both the standard cultivars had poor shoot multiplication with 17.6 µM BAP.

The highest regeneration rate of shoots in almond cv. Nonpariel was obtained on medium containing 1.0 mgl^{-1} BAP + 0.2 mgl^{-1} IBA (Isıkalan *et al* 2011). The enhanced *in vitro* multiplication of Merton I. 793 could be achieved with 0.5 mgl^{-1} BA and 0.01 mgl^{-1} IBA (Soni *et al* 2011).

2.3.3 Rooting of microshoot

Efficient *in vitro* rooting of shoots is a critical step in micropropagation of fruit crops. Losses at this step will have vast economic consequences. The ability of *in vitro* shoots to form roots is affected by several factors, including differences between genotypes (Barros *et al* 2005), culture procedures (Thakur and Kanwar 2008a), mineral nutrition (Liu *et al* 2004), subculture times (Tang *et al* 2006), the level of tissue maturity (Olivier 2004), and physiological age (Tang *et al* 2006). Due to these factors, *in vitro* shoots showing a variety of rooting responses have resulted in plants being variously described as easy-to-root (ETR), or difficult-to-root (DTR) (Marks and Simpson 2000).

Rooting *Pyrus* spp. *in vitro* has proved difficult (De Paoli 1989 and Reed 1995), and scion cultivars have proved more difficult-to-root than rootstocks (Bhojwani *et al* 1984). Adventitious root formation can be induced quite readily in many herbaceous species, but it can be very tough in most woody species. In case of woody species, rooting is the most difficult of various stages to accomplish (Hu and Wang 1983). Zimmerman (1983) reported that for rooting concentration of mineral salts and sugars should be reduced to half or less of the concentration used for proliferation. Auxin is essential for root initiation and NAA is the most effective auxin for induction of root regeneration. Therefore, NAA is mostly used for

inducing rooting followed by IBA, IAA and 2,4-D (Hu and Wang 1983). Lane (1979b) observed that high concentration of auxin induce callus at the shoot base which inhibits normal root development. Thimann (1977) reported that high auxin is undesirable for root elongation phase and hence inhibited by high concentration of auxin.

Chevreau *et al* (1992) successfully rooted European pears, but the results were poorer with Asian pears. In *P. communis* L. cv. Bartlett, Lane (1979c) observed 70 per cent root initiation with 10.0 µM NAA, whereas IBA at the same concentration tended to be toxic. However, at a lower concentration (1.0 µM), IBA was better than NAA. He further noted that conspicuous differences in the type of roots that developed when treated with IBA and NAA. NAA induced roots were thicker and more prolific, whereas those induced by IBA were longer and fibrous. NAA at 10.0 µM concentration inhibited root elongation but it was found advantageous as less number of roots were damaged at the time of transferring the plantlets to soil. Singha (1980) also preferred NAA over IBA for inducing roots in *P. communis* L. cv. Seckel as basal callus developed when IBA was applied even at lower concentrations. Therefore, lower NAA concentrations (2.5-5.0 µM) yielding 40 per cent root initiation was preferred to avoid callus formation.

Viseur (1987) reported that in *P. communis* L. rooting rates on rooting medium containing NAA (1.5 mgl^{-1}) varied with the cultivar. Thirty per cent microcuttings of cv. Professeur Molon formed adventitious roots, 50 per cent in cv. Durondeau, 70 per cent in cv. Conference and 90 per cent in cv. Doyenne du Comice. Upto 80 per cent of microcuttings of *P. communis* L. cv. Bartlett, Pakham's Triumph and Beurre Bosc formed roots on MS medium supplemented with either NAA (10 µM) or IBA (10 µM) (Shen and Mullins 1984). Callus was formed at the base of the cuttings during the first week of inoculation. Adventitious roots emerged from the basal callus during the second week of culture and microcuttings formed upto 20 roots.

Baviera *et al* (1989) observed that NAA caused the formation of basal callus and, with IBA the percentage of rooting was good but the number of roots per plant was lower than IAA in *P. communis* L. cv. Conference. They further reported that the plants with more and bigger leaves rooted better and with a greater number of roots per shoot. Banno *et al* (1988) reported that best rooting for Japanese pear rootstock was in medium containing 1.0 mgl^{-1} IBA. Whereas, Banno *et al* (1989) while working with Japanese pear cultivars reported the best rooting with 2.0 mgl^{-1} IBA.

Differences in rooting ability between two cultivars could be related to differences in auxin uptake and metabolism. Berardi *et al* (1993) analyzed the role of applying IBA in adventitious root formation in microcuttings of two pear cultivars; Conference (easy to root) and Doyenne 'd' Hiver (difficult-to-root). After 4 days of culture the percentage of ^{3}H-IBA taken by shoots was approximately 40 per cent in Conference and 19 per cent in Doyenne 'd'

Hiver. IBA was metabolized very rapidly in both cultivars and 12 hours after application only a small portion of the total extractable radioactivity could be identified as free IBA. Only Conference showed the ability to convert IBA into free IAA during the root induction period. Endogenous level of free IAA rapidly increased in Conference during the first two days, while it remained very low in Doyenne 'd' Hiver.

Al Maarri *et al* (1994) studied the factors affecting the *in vitro* and *ex vitro* root formation in *P. communis* L. cvs. Passe-Crassane and William's Bartlett. No notable differences, in the percentage of rooted cutting with NAA or IBA, in the two cultivars were observed. However, the rooting percentage on medium with IAA was lower. The rooting percentage, number of roots, basal callus formation and the root thickness increased with increasing NAA concentrations, but the average root elongation decreased. The best quality root formation was observed with 0.2 mgl^{-1} of NAA. Bartish *et al* (1994) obtained almost 100 per cent rooting in *P. communis* L. on medium with 0.3-1.0 mgl^{-1} IBA for 2-3 days and subsequent transfer to half-strength MS medium without growth regulators.

Half MS medium supplemented with 2.0 mgl^{-1} IBA was the best for inducing rooting in *P. pashia* (Dwivedi and Bist 1997). In *P. pyrifolia* cv. Gola, Dwivedi and Bist (1999) obtained highest rooting (68.33%) with IBA 1.0 mgl^{-1}. Yeo and Reed (1995) reported that *P. communis* L., OH × F 230 had the highest percentage (>80) of rooting of the three rootstocks, and it rooted well with all IBA and NAA treatments. The best rooting treatment (42.9%) for *P. betulaefolia*, OPR 260 with 10.0 µM IBA in darkness for one week. *P. calleryana*, OPR 157 shoots rooted poorly in all growth regulator combinations with the highest percentage rooting (23.9%) obtained with 10.0 mM NAA dip treatment. In case of five American-bred hybrid pear rootstocks (OH × F 34, 51, 69, 87 and 230) rooting was generally better on the medium containing 2 mgl^{-1} IBA as compared to medium containing 5.0 mgl^{-1} IBA (Nadosy 1997).

Stimart and Harbage (1989) failed to induce rooting in microcuttings of *P. calleryana* cv. Bradford even after 2 years of subculture and intermittent attempts at rooting. In wild pear (*P. syrica*), Shibli *et al* (1997) observed that IBA, IAA and NAA induced *in vitro* rooting and maximum of 72 per cent rooting was achieved with 3.0 mgl^{-1} IAA. Neither activated charcoal nor polyvinyl pyrrolidone influenced *in vitro* rooting. *Ex vitro* rooting was unsuccessful when shoot microcutting were treated with 0.0, 5.0, 10.0 or 15.0 mgl^{-1} IBA, IAA or NAA for one hour and grown under intermittent water mist.

Liaw *et al* (1992) placed shoots of *P. serotina* cv. Hngshan on half-strength MS medium containing phloroglucinol (PG) plus 0.1 or 1 mgl^{-1} IBA and kept in dark for 6 days, before transplanting to auxin-free half-strength MS medium. Sixty per cent shoots rooted plantlets after 30 days. Shoots of *P. kawakamii* cv. Nianli were cultured on half MS medium supplemented with 162 mgl^{-1} PG plus 1.0 mgl^{-1} NAA and kept in dark for 6 days, before transfer to auxin free half MS medium, 70-100 per cent rooted plantlets were obtained after 30 days.

Bhojwani *et al* (1984) found that auxin was essential to induce rooting in the *in vitro* raised shoots of *P. pyrifolia* seedling. Rooting with 2.0 mgl^{-1} NAA was the best of three tested levels of the auxin (0.02, 0.2 and 2.0 mgl^{-1}), but it was also associated with basal callusing in the shoots. Whereas, rooting response of various *P. pyrifolia* cultivars Hosui, Kosui, Nijisseiki, Shinseiki and Shinsui was only 10 per cent. Reed (1995) screened 49 *Pyrus* species and cultivars and one selection of *Pyronia veitchii* for *in vitro* rooting response. Twenty eight accessions, mostly *P. communis* L. cultivars rooted the best (50 %). Rooting varied between 6 and 44 per cent in 13 accessions. However, *P. betulaefolia*, *P. calleryana*, *P. hondoensis*, *P. koehnei*, *P. pashia*, *P. pyrifolia*, *P. regelii*, *P. ussuriensis* and the *Pyronia veitchii* selection failed to root.

Thakur (2004) reported that combinations of NAA and IBA were more effective in improvement of rooting frequency in pear over NAA or IBA alone. In Kainth, Punjab Beauty and Shiara, the highest rooting percentage were obtained on liquid media fortified with NAA and IBA at 0.50 mgl^{-1} each while as in wild pear rooting was on solid media supplemented with NAA and IBA at 0.25 mgl^{-1} each.

Hassanen and Gabr (2012) obtained the highest rooting (85 %) by using half-strength MS medium supplemented with 2.0 mgl^{-1} IBA and the number of roots per shoot was 4.0 and root length reached 3.0 cm.

Peer *et al* (2013) reported that MS medium fortified with IBA (2.50 mgl^{-1}) caused highest rooting (86.33 %), maximum root length (44.33 mm) and maximum root number per explant (4.90) in sweet cherry cv. Bigarreau Noir Grossa.

A protocol was developed for *in vitro* rooting of *P. communis* L. rootstock by Previati *et al* (2002) who found that rooting was in general higher in MS medium than with QL (Quoirin and Lepoivre) medium. They also found that the concentration of macronutrients was ineffective in MS, while for QL the 50 per cent concentration resulted better than 100 per cent concentration.

Most workers used MS medium, full-strength or diluted, for the rooting phase in pears. However, Ochatt and Caso (1984) found liquid medium to be more efficient than agar-containing medium. Marino (1984) also tested a short liquid pre-treatment in IBA (1000 µM) followed by transfer to solid hormone-free medium. A good rooting percentage was obtained by following this method, but the survival of plants during acclimatization was lower than that obtained after induction on solid medium containing auxin. Hirabayashi *et al* (1987) found liquid IBA pre-treatment (1000 µM), followed by transfer to sterile vermiculite plus MS medium, to be the more efficient technique for rooting of Asian pear variety Hosui.

2.4 Shoot tip grafting/ Micrografting

Grafting is commonly used to propagate several plant species. However, conventional grafting has a limited scope due to the lack of high humidity conditions, risk of bacterial or

fungal contamination or unreliable grafting techniques. In order to overcome these problems, *in vitro* micrografting is an alternative. This technique has several advantages not only for propagation purposes but also for research (Ramanayake and Kovoor 1999, Estrada-Luna *et al* 2002, Amiri 2006, Dobranszki and Jambor 2006, Hassanen 2013).

The micrografting technique can provide an efficient production of high quality true-to-type plants in a short time period and under controlled and aseptic conditions. Moreover, it is possible to produce large quantity of plant material in reduced physical space (Lambardi *et al* 1997). The first attempt on micrografting was made in citrus by Murashige *et al* (1972) and was studied in detail by Navarro *et al* (1975) who developed a standard procedure for obtaining successful micrografts. The morphological and anatomical characteristics of both compatible and incompatible combinations in pear were studied by Musacchi *et al* (2004) and reported that high callus proliferation of the compatible cv. Beurré Hardy with quince MC and clonal seedling OH × F40, while as incompatible cv. Beurré Bosc showed no activity until 4 days after grafting.

Espen *et al* (2005) studied differential and functional connection of vascular elements in compatible and incompatible pear/quince internode micrografts. Micrografts of internodes excised from *in vitro* grown pear [*Pyrus communis* L. cv. Bosc (B) and cv. Butirra Hardy (BH)] and quince (*Cydonia oblonga* cv. Malling Clone (EMC)] were cultured aseptically and it was found that incompatible heterograft (B/EMC) showed a marked delay in internode cohesion compared with autografts (B/B and BH/BH) and compatible heterograft (BH/EMC).

Musacchi *et al* (2004) while working on *in vitro* grafting of pear/quince revealed that *in vitro* grafting performs well, with graft-take of around 100 per cent in the compatible unions after 60 days. They further reported that most suitable stage of plant development for micro-graft to guarantee good results is when stem is 2-3 mm thick and diameters of rootstock and scion must be similar to ensure successful graft-take.

The work on micrografting of fruit plants has been conducted successfully by various workers (Dobranszki *et al* 2005, Nunes *et al* 2005, Can *et al* 2006, Hsina and El Mtili 2009, Aazami and Hassanpouraghdam 2010, Rafail and Mosleh 2010, Isikalan *et al* 2011, Isaia *et al* 2011, Hassanen 2013).

2.4.1 Scion origin

The origin of the scion can greatly affect the micrografting success. Deogratias *et al* (1991) studied *in vitro* micrografting of apricots by using actively growing or dormant shoot tips (0.5-1.0 mm) from different sources: (a) dormant buds collected from November to February (b) vegetative flushes from plants growing in the field (c) *in vitro* derived vegetative shoots. The effects of rootstock cultivar, position of the shoot tip on the rootstock, shoot tip size, light and temperature, composition of the medium and growth regulator treatments on the success of grafting on rootstock seedlings growing *in vitro* were studied. The best source

of shoot tips was found to be *in vitro* derived microshoots while, dormant buds collected in November proved inferior one. Many of the grafts became quiescent after initial growth.

Hassanen (2013) also reported the highest grafting success while using *in vitro* scion source and obtained grafting success of 83 per cent by using *in vitro* scion (>0.5 cm but <1 cm) of Le Conte pear on *in vitro* decapitated *P betulaefolia* as rootstock.

Bhatt (2008) also reported that the best source of shoot tips was from *in vitro* derived shoots and the poorest source was from *in vivo* forced shoot tips in apple. Higher contaminated cultures (39.04 %), browning/necrotic cultures (57.15 %) and browning/ necrosis intensity (3.38) was observed when scions derived from *in vivo* forced tips were used for grafting which decreased to 10.80 per cent, 39.06 per cent and 2.20, respectively when *in vitro* derived scion tips were used.

Dziedzic and Malodobry (2006) carried out micrografting using cherry cultivar Regina on rootstocks Damil and Gisela 5 and cv. Van on rootstock Damil. Shoot tip explants of Regina and Van were 0.2 – 0.3 and 0.4 – 0.5 cm in length, respectively. Different pre-treatments of scions and stocks, ways of grafting and light conditions after grafting influenced the graft success. Before grafting, the upper part of the rootstocks and the lower end of scions were dipped for one minute in different antioxidant and growth regulator combinations. There was no browning of scion and stocks cut surface following these treatment combinations. Treatment combination of 150 mgl^{-1} citric acid + 0.1 mgl^{-1} GA_3 +0.5 mgl^{-1} IBA resulted in higher graft success.

Baydar and Celik (1999) used *in vitro* micrografting technique in grapes. Shoot tip meristems of Kalecik Karasi, Emir, Uslu, Hafizali and Razaki grape cultivars were micrografted onto Kober 5BB seedlings. Shoot tip meristems were obtained from *in vivo* (shoots from actively growing vines in the vineyard or shoots from one-year-old cuttings in the greenhouse) or *in vitro* (shoots obtained by shoot tip culture in aseptic conditions) sources. The micrografted plantlets were cultured on solid MS medium for 2 months. The micrografting success rate was highest using *in vitro* shoot tips, varying from 40.6 per cent in cv. Hafizali to 68.3 per cent in cv. Emir.

Ghorbel *et al* (1998) performed *in vitro* grafting of almond cv. Achak on aseptic rootstocks in order to estimate the success of micrografting in relation to the mode of grafting, the scion origin and the physiological state of the grafts. Both active and dormant small shoots, buds and meristematic apices were collected from field plants. In this study, 4-6 week old shoots and small apical buds (3.0-5.0 mm) formed *in vitro* were also used. These shoots and buds grafted on top or in tongue-grafts gave a higher percentage (82.1 and 79.2 %) respectively of successful grafts. Contamination occurred more often in material taken from the field and necrosis was observed frequently when physiologically active meristematic apices were used. Similarly, Onay *et al* (2004) reported that the best growth of microscion in

Pistachio was obtained with the *in vitro* shoot tips rather than with shoot tips excised from a field grown tree.

Sanjaya *et al* (2006) were successful in achieving *in vitro* micrografts of sandal wood (*Santalum album* L.) by placing 1-2 cm long scions derived from nodal segments onto the hypocotyls of 45-day-old *in vitro* rootstocks. Use of *in vitro* grown shoots as a source of scion gave better graft success (60%) than scions collected directly from field grown trees. *In vitro* grafting was also influenced by scion size and root age.

2.4.2 Scion length

Hassanen (2013) reported successful micrografting of pear cv. Le Conte on *in vitro* decapitated *P betulaefolia* as rootstock. Shoot tips consisted of two different sizes, <0.5 cm long and >0.5 cm but <1 cm long were examined. The highest percentage of successful grafts, axillary shoots development percentage, micrograft length increase and number of new buds formation (83 %, 80 %, 3.5 cm and 4 cm, respectively) were obtained with scion length greater than 0.5 cm, while the scion of smaller than 0.5 cm was achieved 37 % of successful grafting.

It is necessary to choose a shoot tip of size that gives a realistic degree of grafting success. Onay *et al* (2004) carried out *in vitro* grafting in *Pistachio* and studied different variables including different size of microscion, grafting methods, effect of culture medium and effect of time of the year at which shoot tips were used. The results indicate that the easiest and most successful method for grafting was slit micrografting. Higher levels of micrograft take were achieved with 2-4 mm (56.76 %) and 4-6 mm (79.25 %) long scions obtained from the regenerated shoot tips.

Bhatt (2008), while working on micrografting of Lal Ambri apple on M-9 rootstock, observed that graft success increased with the size of scions up to 10 mm, then decreased with increase in size. Graft success of 12.92 per cent was obtained when scion tips of 10 mm were used for grafting which decreased to 10.53 and 8.94 per cent when scion tips of 5 and 15 mm were used, respectively.

Amiri (2006) studied the effect of grafting method, scion size and its origin on grafting success. Grafting success was significantly dependent on the method of grafting and size of the scion. One month old *in vitro* cultured meristematic apices with length of 5-15 mm were used as microscion. The highest percentage (65 %) of successful grafts was obtained for homoplastic apex graft (shoot tip), with apical bud scion length greater than 6 mm. Whereas, the lowest percentage (16 %) of success was observed in the heteroplastic side bud apices (wedge) grafts. As the size of the microscion increased (from 3 to 15 mm), the success rate of the grafts improved and reached 34 to 65 per cent. Khalafalla and Daffalla (2008) noticed that rate of successfully grafted shoots in Gum Arabic tree was influenced by both scion length and rootstock age. Success rate was 100 per cent with scion length of 3.0 cm and rootstock of 14 days.

Thimmappaiah *et al* (2002) observed that length of scion shoot had significant effect on micrografting success. Graft success was higher (79.5 %) when the scion length was greater than 5 mm and it was lesser (0.5 %) when size of scion was smaller (3-5 mm) in cashew.

2.4.3 Media

Modification of basal medium formulation has been an effective means for achieving graft success. The sucrose concentration of the nutrient medium of grafted plants has been found to play a significant role.

Hosoi *et al* (1979) reported that sucrose concentration in the liquid medium significantly affected the percentage success in citrus and best results were obtained with 45 gl^{-1} sucrose. Gebhardt and Goldbach (1988) described micrografting technique of *Prunus* shoots derived by shoot tip culture. Autografts of plum cv. Hauszwetsche as well as heterografts of several sour cherry cultivars (Schattenmorelle, Weiroot 158 and Koroser) were established. A mechanically strong graft union was formed during the course of a three week subculture of micrografts in a liquid medium without the addition of growth regulators. Deogratias *et al.* (1991) obtained best results in micrografting of apricot when cultured in a medium comprising MS salts with 3 per cent sucrose.

Abousalim and Mantell (1992) carried out study on micrografting in *Pistachio cv.* Mateur, on the rootstock of the same cultivar. The scion bases of cv. Mateur were cut into gently sloping wedges and were inserted into vertical splits in the cut surface of the epicotyls. Micrografted seedlings were cultured on liquid MS medium using a filter paper bridge. In second experiment, surface disinfected seeds were cultured directly on top of a Milcap-synthetic (polypropylene fibre) support and after 12 days, the seedlings were micrografted *in situ* with scions. The best results were obtained using the later method and vascular connections were established across grafts within 21 days. Axillary shoots were produced by 60 per cent of the scions. The Milcap -polypropylene supports allowed good growth and branching of root system and prevented root damage during grafting.

Grewal *et al* (1994) successfully cultured shoot tip grafted plants of various cultivars of citrus in a liquid medium containing the salts of MS medium, the vitamins of White's medium and sucrose (7.5 %). Baydar and Celik (1999) micrografted several grape cultivars onto Kober 5BB seedlings. These micrografted plantlets were cultured on Murashige and Skoog (MS) medium for two months. Banerjee *et al* (2000) developed an efficient and highly reproducible *in vitro* micrografting protocol for microshoots of cotton (*Gossypium hirsutum* L.). Culture of grafted shoots in Murashige and Skoog's basal liquid medium devoid of growth regulators showed maximum rate of survival (95 %), elongation of scions and number of nodes per plant.

Thimmappaiah *et al* (2002) developed a successful micrografting technique in cashew using *in vitro* germinated seedlings as rootstocks and axenic shoot cultures (shoot tip and nodal cultures) established from mature tree as microscions. *In vitro* germinated seedlings which emerged 20-25 days after inoculation on absorbent cotton were decapitated and used as rootstock. Mature tree explants initiated on hormone-free Murashige and Skoog (1962) modified medium were prepared as scions of 3-15 mm length for grafting. Micrografts were successfully cultured on hormone free liquid half MS medium and potted out after 10-12 weeks of culture growth.

Dziedzic (2004) carried out micrografting of cv. Regina on Gisela 5 rootstock on two types of MS medium prepared as liquid and semi-solid with 0.7 per cent agar. High per cent of successful micrografts were achieved on semi-solid MS medium. Higher contaminated cultures were observed in liquid media. However, vigorous plantlets developed in this liquid media. Naz *et al* (2007) carried study to assess the potential use and applicability of micrografting technique for the development of virus free nursery in citrus. Some techniques that tend to increase the grafting success were employed. MS medium fortified with 3, 5 and 7 per cent sugar was used in combination with two grafting methods *viz.* inverted-T incision and surface placement in Kinnow mandarin and Succari sweet orange. Grafting was carried out under aseptic conditions by using 15 day old etiolated seedlings of rough lemon as rootstock. Shoot tips (1-2 mm) and 3 leaf primordia (0.3 – 0.5 mm) were taken from the fresh shoot flushes and grafted *in vitro.* Higher grafting success of 34.7 per cent was recorded with inverted-T incision than surface placement which gave 26.7 per cent successful micrografts. A total of 21 per cent successful micrografts were achieved at 3 per cent sugar level which increased significantly to 33 per cent with increase in sugar level to 5 per cent in both cultivars.

2.4.4 Graft method

The method of placement of the excised shoot tip onto the rootstock significantly influences the grafting success. Various methods of placement of scion tip onto rootstock have been described. Abreu *et al* (2003) studied micrografting of tissue cultured apple plants. Scions were cleft-grafted on the rootstocks of *Malus pumila* and *M. prunifolia*. Graft development resulted in the generation of meristem tissue with parenchymatous cells originating at the graft interface and cambial tissue proliferation from the scion. This allowed a vascular system connection between the scion and rootstock, resulting in micrograft viability.

Bhatt (2008) observed that vertical slit grafting as best method for carrying out micrografting in apple. He reported highest graft success (23.83 %) and lowest browning/ necrotic cultures (29.33 %) with vertical slit method. The graft success decreased to 21.00 per cent and 13.10 per cent and browning/necrosis incidence per cent increased to 34.68 per cent and 40.36 per cent when scion was grafted by wedge and horizontal placement, respectively.

Onay *et al* (2004) studied the influence of different grafting methods on graft success in *Pistachio*. He observed that the slit method was easiest and most successful one giving success of 80 per cent whereas 60 per cent graft success was obtained under wedge micrografting. In vertical slit micrografting no problem was observed during the union formation as long as the contact surfaces were perfectly smooth.

Dobranszki *et al* (2000a) described the requirements for *in vitro* micrografting in apple cv. Royal Gala which served as source of both rootstock and scion. Oxidative browning of cut surface was inhibited by the use of antioxidant mixture during grafting process. Scion base of Royal Gala cut in V-shape was dipped in 1.0 per cent agar-agar solution and stuck into the vertical slit of rootstock. There was no displacement and the rate of fused and further developed grafts were 95 per cent.

Naz *et al* (2007) investigated the applicability of micrografting technique for the development of virus free nursery in citrus and reported that success in micrografting varies with the method and plant species or genotype. Graft success of 34.7 per cent was recorded with inverted-T incision whereas; surface placement gave 26.7 per cent successful micrografts. Succari sweet orange responded maximum with surface placement method whereas, Kinnow mandarin showed better response with inverted T incision.

Toth *et al* (2006) studied the efficiency of *in vitro* grafting method of six plant species-pepper, melon, apricot, peach, tobacco and chrysanthemum. Plants with short roots were removed from the media and the stem of the scion and the stock (at the four to five leaf stage) were cut at right angles, each with 2-3 leaves remaining on the stem. The stem of the scion was cut in a wedge with scalpel in Petri-dish and the tapered end was fitted into a cleft made in the cut end of stock. The graft was firmed with silicon rubber clip hauled up with dentist's forceps. The survival rate of the grafted plants was excellent in case of herbaceous plants (90-100 %), whereas it was 30 per cent for woody species.

Dziedzic and Malodobry (2006) carried out micrografting using cherry *cv*. Van and Regina on Damil rootstock using different methods of grafting. The wedge shaped end of scion 'Van' was inserted into 0.5-0.6 cm slit in rootstock Damil, whereas, even end of shoot tip of Regina was placed on Damil rootstock. The grafts were placed individually into test tubes on WPM medium, with 30 gl^{-1} sucrose and agar 7 gl^{-1}. High per cent of successful grafts were obtained for cleft grafting method than shoot tip grafting. The scions and stocks were attached to each other closely and the per cent of graft unit displacement was low.

Wu *et al* (2007) developed shoot tip micrografting technique in *Protea cynaroides* wherein, seedlings were decapitated and a vertical incision made from the top end. The axenic microshoots were cut into a wedge (V) shape and placed them into the vertical incision of the rootstock. Best results were obtained by placing the microscions directly onto the rootstocks without any pre-treatment. Hassanen (2013) also reported that grafting success was

significantly influenced by grafting method employed during micrograting of pear cv. Le Conte on *P betulaefolia*.

2.4.5 Pre-treatment of growth regulators

The success of micrografting to obtain disease-free plant material depends on various factors. Among these factors, pre-treatment with growth regulators have resulted in speeding up the healing of tissue at graft union, resulting in higher survival of grafts. The use of pre-treated apices not only markedly increases the grafting success but also overcome problems encountered during the handling of *in vitro* micrografting that results in browning and drying of apices. Together with auxins, cytokinins stimulate both apex initiation and the union between rootstock and scion by increasing vascular bundle formation (Quoirin *et al* 1977). Cytokinins rejuvenate plant cells and stimulate cell proliferation of graft union tissue with auxins (Bessis 1986).

Bhatt (2008) reported treatment of Lal Ambri microscions with combination of Kinetin and 2,4-D at 0.5 and 5.0 mgl^{-1}, respectively before grafting on M-9 showed highest graft success (42.25 %) and lowest browning/ necrosis incidence (19.00 %) and browning/necrosis intensity (0.20).

Starrantino *et al* (1986) reported that best results in citrus were obtained when both components of graft were pre-treated with 0.5 ppm BA, 0.5 ppm kinetin or 10.0 ppm 2, 4-D. Lemons gave the highest successful grafts (88-92 %) and Clementines the lowest (63-73 per cent).

Edriss and Burger (1984) studied the various factors affecting the success of shoot tip grafting. Growth regulator pre-treatment of scions with 2, 4-D and kinetin was accomplished by dipping the shoot apex of 'Mexican' lime, 'Valencia' orange and 'Star Ruby' grape fruit in growth regulator solutions just prior to excision of the apical dome and primordial leaves. Dipping the shoot tip in 2, 4-D (10.0 mgl^{-1}) or kinetin (1.0 mgl^{-1}) before grafting nearly doubled the success rate compared to control. High concentration of kinetin (10.0 mgl^{-1}) and/or low concentration of 2, 4-D (1.0 mgl^{-1}) were no more effective than the control. There were no differences among the three rootstocks in their response to pre-treatments.

Jonard *et al* (1987) reported that inserting an agar block containing different plant growth regulators resulted in 100 per cent successful *in vitro* grafts of Eureka lemon on Troyer citrange. Apex pre-treatment on Murashige and Skooog (1962) medium plus 3 per cent sucrose with GA_3 at 2.9 mmoles/litre, BA at 44 mmoles/litre and 2, 4-D at 0.009 mmoles/litre.

Starrantino and Caruso (1988) carried out micrografting experiments in citrus species with several growth substances and observed that dipping the apex and the decapitated seedlings for 10 minutes before micrograting in a solution of BA (0.5 ppm) increased the percentage rate of sprouting from 73 to 91 per cent.

Parthasarathy *et al* (1997) conducted experiments to standardize procedures for *in vitro* grafting of Khasi mandarin on various rootstocks. The immersion of Khasi mandarin shoot tips in 2,4-D (10 μgl^{-1}) for five minutes before grafting improved the grafting success on Cleoptara mandarin, Rangpur lime, Kagzi lime and Khashi mandarin. Histological studies using a scanning electron microscope showed callus development between rootstock and scion. Good graft union and complete vascular connection between the shoot tips and the rootstocks were achieved.

Kumarin *et al* (2000) reported the influence of pre-treatments of growth regulators on the success of graft take in Nagpur mandarin cultivar on rough lemon rootstock. Just prior to grafting, both rootstocks and scions were dipped in growth regulator solutions for 10 minutes. The solutions tested were BA (0.5, 1.0, 2.0 and 5.0 mgl^{-1}), kinetin (0.5, 1.0, 2.0 and 5.0 mgl^{-1}) and 2, 4-D (1.0, 2.0, 5.0 and 10.0 mgl^{-1}). Grafting success was highest with Kinetin (1.0 mgl^{-1}) or 2, 4-D (10.0 mgl^{-1}).

Nunes *et al* (2005) studied the micrografting technique in apple by using *in vitro* plants and verifying the influence of application of growth regulators IBA and BAP in Gala/M-9 and Gala/Marubakaido as scion rootstock combinations. The percentage of viable micrografts for the connection of vascular tissues, varied from 73 to 93.2 per cent. The percentage of viability observed highest in Gala/M-9 (93.2 %) on half-strength MS medium supplemented with 2.2 mM IBA, followed by half-strength MS medium pre-treatment with IBA (4.0 mM) in both combinations showed (86.4 %) viability. Results showed that treatment with growth regulators at the point of grafting significantly increased the viability of the graft.

2.4.6 Vitrification

Micropropagation of woody plants is often hampered by the phenomenon of hyperhydricity or vitrification (Chakraborty *et al* 2005). Hyperhydricity, which is characterized by a glassy or swollen appearance to the tissue, usually results in reduced multiplication rates, poor quality shoots and tissue necrosis (Ziv 1991). The so-called vitrified, vitreous or hyperhydric shoots appear turgid, watery at their surface and hypolignified. Their organs are translucent and in some cases less green and easily breakable.

Occurrence of hyperhydricity remains unpredictable and many factors appear to induce hyperhydricity (Kevers *et al* 1984). Stress is believed to be the major factor underlying the phenomenon. Stress during *in vitro* culture could arise as a result of wounding, the properties of the tissue culture medium, infiltration of tissue by the culture medium which is generally of a higher ionic strength and rich in nitrogen or environmental conditions during *in vitro* culture in sealed containers (Deberg, 1983). This stress could mediate a rapid endogenous ethylene burst. One of the reasons of poor success rate in micrografting is vitrification.

Bhatt (2008) reported that media formulations significantly influences vitrification percentage of apple. Lowest vitrified cultures (4.94 %) were obtained in MS semi- solid

(using 5.0 gl^{-1} agar) media with 6 per cent sucrose which increased non-significantly to (6.29 %) in the same media but with 3 per cent sucrose. Highest vitrified cultures (16.32 % and 12.58 %) were observed in liquid medium at 3 and 6 per cent, sucrose respectively. MS liquid medium plus vermiculite also showed significantly higher vitrified cultures than obtained in MS semi solid media.

Amiri (2006) reported that initial percentage of successful grafts of cherry (*Prunus avium* L.) var. Seeyane Mashad was about 75 per cent which decreased at the end of experiment. The poor success rate was mainly due to excessive collogenesis observed on more than 50 per cent of the apices and vitrification on more than 30 per cent of the explants.

Al-Maarri and Al-Ghamdi (1996) noticed that the frequency of vitrification increased on medium containing a low concentration of sucrose. Ghorbel *et al* (1998) studied *in vitro* grafting of almond cv. Achak performed on asceptic roots in order to estimate the success of micrografting in relation to the mode of grafting, the scion origin and physiological state of grafts. He achieved low success rates due to vitrification in more than 70 per cent of explants. Dziedzic (2004) carried out micrograting experiments in cherry cv. Regina on Gisela-5 rootstock on two types of Murashige and Skoog medium: liquid medium and semi-solid agar medium. High per cent of successful micrografts were obtained on MS semi-solid medium. Use of liquid medium resulted in maximum vitrified plantlets.

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CHAPTER III

PUBLISHED/ ACCEPTED/SUBMITTED RESEARCH ARTICLES

Sr. No	Publication	Journal	NAAS Score	Status
1	*In vitro* propagation of Patharnakh (*Pyrus pyrifolia* (Burm F.) Nakai) pear	Research Journal of Agricultural Sciences	3.51	Published ISSN: 0976-1675
2	Micropropagation of Kainth (*Pyrus pashia*) - an important rootstock of pear in northern subtropical region of India	Journal of Experimental Biology and Agricultural Sciences	Under assessment	Published ISSN: 2320-8694
3	Micropropagation of Patharnakh (*Pyrus pyrifolia* (Burm f.) Nakai) pear using explants obtained from forced cuttings	International Journal of Agricultural Sciences and Veterinary Medicine	Under assessment	Published ISSN:2320-3730
4	*In vitro* propagation of Kainth (*Pyrus pashia*) using explants from forced cutting	Pakistan Journal of Botany	6.87	Submitted Confirmation mail is attached ISSN: 0556-3321

3.1

Research Paper

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In vitro Propagation of Patharnakh (*Pyrus pyrifolia* Burm F. Nakai) Pear

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ABSTRACT

In vitro propagation of Patharnakh (*Pyrus pyrifolia* Burn F. Nakai) pear was carried out in Tissue Culture Laboratory in Department of Fruit Science, Punjab Agricultural University, Ludhiana (Punjab) during 2011-13. Nodal explants from Patharnakh were used for their *in vitro* propagation protocol development. The effect of various media {½MS (M_1), MS (M_2) and WPM (M_3)} and growth regulators (BAP, IBA and NAA) on establishment, proliferation and rooting was studied. Lowest necrotic culture percentage was observed by using M_2 medium fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum establishment (96.10%) was obtained on M_3 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum proliferated cultures (79.47%) were obtained using M_3 medium fortified with BAP (5.0 mgl^{-1}). However, maximum shoots per explant (3.08) were produced in M_2 medium supplemented with BAP (2.5 mgl^{-1}). Shoots of maximum length (53.75 mm) were obtained using M_3 medium containing BAP (0.5 mgl^{-1}). Rooting was maximum (9.16%) using M_1 medium fortified with IBA (1.0 mgl^{-1}) while as no rooting was obtained irrespective of media using NAA. Roots per explant were 2.60 using M_1 medium supplemented with IBA (1.0 mgl^{-1}). However, roots of maximum length were obtained using M_3 medium supplemented with IBA (1.0 mgl^{-1}).

Key words: *Pyrus pyrifolia*, *In vitro* propagation, Necrotic culture, Shoot proliferation, *In vitro* rooting

The domestic pears of Asia are divided mostly from *Pyrus pyrifolia* Burm F. Nakia, known as Japanese or sand pear. The sand pear, *Pyrus pyrifolia* is the main cultivated species in southern and central China and in Japan, but *Pyrus ussuriensis* Maxim, is also grown in north China and Japan (Shen 1980). Hybrids of *Pyrus communis* and *Pyrus pyrifolia* are grown in some parts of North America. Presently pear is next to apple in importance, acreage, production and varietal wealth among temperate fruits in India. Pear has become an important fruit crop of Punjab. Current pear production relies on a few numbers of main cultivars of Patharnakh (hard pear) which is the leading cultivar of Punjab followed by Baggugosha and Leconte.

Since, the fruiting characteristics of plants produced from seeds are nearly always inferior to the parent plant due to heterozygosity, thus their seed progeny is not true to type (Jackson and Looney 1999). Asexual propagation on the other hand gives rise to plants which are genetically identical to the parent plant and thus permits the perpetuation of the unique character of the cultivar. Tissue culture techniques are becoming increasingly popular as alternative means of plant vegetative propagation. In recent years, tissue culture techniques (micro-propagation) are increasingly used for rapid clonal multiplication of several economic plants, restoration of vigour and yield lost due to infection and preservation of germplasms. *In vitro* propagation has shown promises for rapid and large scale clonal multiplication of disease free planting plant throughout the year. *In vitro* propagation has been reported in several pear cultivars viz Rocha (Friere *et al*. 2002), Gola (Dwivedi and Bist 1999), Conference (Baviera *et al*. 1989), Seckel (Singha 1982), Durondeau (Lucyszyn *et al*. 2006) and Sebri Karimpour *et al.* (2013).

MATERIALS AND METHODS

Patharnakh plants growing in New Orchard, Department of Fruit Science, Punjab Agricultural University, Ludhiana (Punjab) were used as a source of explants in the present study. Nodal segment explants were taken from current season's growth from March to November. The explant source was subjected to uniform cultural practices. The basal media used in the study were Murashige and Skoog's medium with half strength of macro and micronutrients (M_1), Murashige and Skoog's medium (M_2) and woody plant medium (M_3). The basal media were modified by adding measured quantity of growth regulators. The pH of medium was adjusted to 5.8 with pH meter using 1N HCl or 1N NaOH before heating. Agar at the rate of 7.5 gl^{-1} was dissolved by placing medium on gas burner. The medium was autoclaved at 15 psi and 121°C for 15 minutes.

Media were allowed to solidify at room temperature. Explants were first washed in running tap water for 15 minutes followed by keeping in 1 per cent bavistin along with few drops of tween-20 for 20 minutes. Later on explants were washed thoroughly by keeping under running tap water till all residues gets washed out. Before culturing, explants were sterilized with 0.1 per cent $HgCl_2$ for 5 minutes within laminar air flow cabinet, followed by 3-4 washing using autoclaved distilled water. Surface sterilized explants were transferred to sterilized Petri dish using sterilized forceps. The explants were inoculated in test tubes/glass jars containing autoclaved media using sterilized forceps for establishment. Different media (½ MS, MS and WPM) fortified with different combinations of 6-benzylaminopurine (BAP) {0.5-1.5 mgl^{-1}} and indolebutyric acid (IBA) {0.01-0.5 mgl^{-1}} were used during establishment stage. The data were recorded on necrotic cultures (%) and explant establishment (%) after 3 weeks of inoculation.

The explants which got established on establishment medium were used as material for proliferation. The established explants were taken out aseptically from culture vessels using sterilized forceps. Necessary dissection of established explants was carried out using sterilized blade and forceps before transfer to shoot proliferation media. Various shoot proliferation media i e ½ MS, MS and WPM fortified with various concentrations of BAP i e 0.5-3.0 mgl^{-1} were used. The optimum media and concentration of BAP for shoot proliferation was standardized. Observations on proliferated cultures (%), number of shoots/explant and average length of shoot (mm) was recorded after third subculture and the culture duration were five weeks each. After allowing shoots to multiply on shoot proliferation medium, individual shoots were separated (2cm) and transferred to root regeneration medium. Different types of media i e ½ MS, MS and WPM containing various combinations of IBA (0.5-2.0 mgl^{-1}) and NAA (1-Naphthaleneacetic acid) {0.5-2.0 mgl^{-1}} were used. Observations on rooting (%), number of roots/explant and average length of roots (mm) were recorded four weeks after culturing. The data generated in course of the present study was analyzed using CRD (factorial), replicated at least 3 times.

RESULTS AND DISCUSSION

Effect of medium supplemented with growth regulators on necrotic culture (%)

The data of the present investigation clearly shows that both media and growth regulator combination had significant effect on necrotic culture induction during establishment stage. Least necrotic culture (10.58 %) was observed on M_2 medium followed by 16.59 per cent on M_3 medium and 20.10 per cent on M_1 medium (Table 1). All media differ significantly from each other in terms of induction of necrotic cultures. As for as growth regulator combination was concerned, BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved best with least necrotic culture percentage of 11.76 followed by 12.19 per cent on BAP (1 mgl^{-1}) and IBA (0.5 mgl^{-1}). Interaction studies revealed that the lowest necrotic culture of 2.04 per cent were induced by M_2 supplemented with BAP (1 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by 6.00 per cent on M_2 fortified with BAP (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}). Highest necrotic culture (27.00%) was induced by M_1 fortified with BAP (1.5 mgl^{-1}) and IBA (0.5 mgl^{-1}). These results are in conformity with earlier observations made by De-Paoli (1989), Liete *et al*. (1997), who reported Murashige and Skoog medium as the best medium for culture initiation resulting in the highest establishment (%) with least necrotic cultures. Higher necrosis in Woody Plant Medium may be attributed due to lower total nitrogen content (Mamaghani *et al*. 2010). Literature on induction of necrotic culture during establishment stage is scanty and inconclusive. Difference in induction of necrotic response in different media tested might be due to difference in composition of basal media.

With regard to growth regulator combination, various scientists have reported different growth regulator combination but variation observed under present study may be due to genotypic difference (Karimpour *et al*. 2013). Berardi *et al*. (1992) studied the effect of auxins (IBA and NAA) on incidence of shoot tip necrosis during *in vitro* rooting of *P. calleryana*. They found that shoot tip necrosis was not influenced by auxins. Salisbury and Ross (1992) reported that plant species-specific cytokinin to auxin ratio has role in establishment, proliferation and growth of explant. Despite considerable research on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their *in vitro* effects (Bairu *et al*. 2009). Influence of plant growth regulators on explant necrosis might be influenced by medium composition, growing conditions and genotype (Bairu *et al*. 2009).

Effect of medium supplemented with growth regulators on explant establishment

Perusal of data (Table 2) revealed that highest explant establishment of 52.65 percent was obtained on M_2 medium followed by M_3 and M_1 medium. BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved the best combination for explant establishment followed by BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}). Interaction studies revealed that the highest explant establishment of 96.29 per cent was obtained on M_2`medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) which was statistically best than rest of treatments (Fig 1). Lowest explant establishment was obtained on M_1 medium supplemented with BAP (1 mgl^{-1}) and IBA (0.25 mgl^{-1}). Different media have been tried earlier for establishment of various plant species by various workers and reported varied results in terms of establishment percentage. Peer *et al*. (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir Grossa using MS medium. Similar results were obtained by Dai-Hong *et al*. (2004), Sedlak *et al*. (2008), Hassanen and Gabr (2012), Thakur and Kanwar (2008a). However, variations in establishment (%) observed in terms of growth regulator combination from earlier studies might be due to different genotype, growing conditions, physiological state and

cytokinin to auxin ratio of explant (Karimpour *et al*. 2013). Difference in establishment (%) in various media at different combinations of BAP and IBA may be attributed to varying callusing rates, necrosis and contaminations values (Kozlina and Jelaska 1987). Rathore *et al*. (1991) also reported higher establishment of explants in M_2 medium (MS) supplemented by low level of auxin (IBA) and comparatively higher levels of cytokinin (BAP) in various forest trees.

Table 1 Effect of media type and growth regulators (mgl^{-1}) on necrotic cultures (%)

Growth regulator combination (mgl^{-1})	Media			Mean
	M_1 (1/2 MS)	M_2(MS)	M_3(WPM)	
BAP (0.5) + IBA (0.01)	23.50	14.12	19.41	19.01
BAP (0.5) + IBA (0.25)	19.39	8.19	16.15	14.58
BAP (0.5) + IBA (0.5)	25.06	6.00	15.28	15.45
BAP (1.0) + IBA (0.01)	16.16	2.04	17.08	11.76
BAP (1.0) + IBA (0.25)	19.31	16.15	18.62	18.03
BAP (1.0) + IBA (0.5)	15.69	8.64	12.25	12.19
BAP (1.5) + IBA (0.01)	12.67	16.83	17.21	15.57
BAP (1.5) + IBA (0.25)	17.65	11.49	14.47	14.54
BAP (1.5) + IBA (0.5)	27.00	14.05	18.50	19.85
Control	24.53	8.30	16.93	16.59
Mean	20.10	10.58	16.59	
C.D ($p \leq 0.05$)	Media (A) = 0.475, GR's (B) = 1.132, A × B = 1.933			

Fig 1 Establishment of Patharnakh using M_2 fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1})

Fig 2 Proliferation of Patharnakh using M_3 fortified with BAP (5.0 mgl^{-1})

Table 2 Effect of media type and growth regulators (mg/l) on explant establishment (%)

Growth regulator combination (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP (0.5) + IBA (0.01)	28.18	75.20	18.72	40.70
BAP (0.5) + IBA (0.25)	38.20	57.45	18.74	38.13
BAP (0.5) + IBA (0.5)	23.56	59.43	14.40	32.46
BAP (1.0) + IBA (0.01)	26.37	70.71	42.81	46.63
BAP (1.0) + IBA (0.25)	0.76	18.34	8.30	9.13
BAP (1.0) + IBA (0.5)	9.11	42.14	25.07	25.44
BAP (1.5) + IBA (0.01)	63.7	96.29	90.67	83.55
BAP (1.5) + IBA (0.25)	52.16	52.78	73.46	59.47
BAP (1.5) + IBA (0.5)	34.65	38.73	66.52	46.63
Control	9.18	15.44	6.16	10.26
Mean	28.59	52.65	36.49	
C.D ($p \leq 0.05$)	Media (A) = 0.852, GR's (B) = 1.556, A × B = 2.695			

In vitro shoot proliferation

Shoots from established explants were cultured on different media supplemented with varying doses of BAP. Initially, the shoot growth was very slow in all media at all levels of BAP. However, after two subcultures on respective media, two to three axillary shoots arose from the base of cultured shoots. From the results it is clearly visible that proliferated culture (%) was highest in M_3 medium (56.85%), which is significantly higher than 51.28 percent in M_2 and 51.11 percent in M_1 medium (Table 3). Proliferated culture (%) increased significantly with increase in concentration of BAP. There was significant interaction

between type of media and BAP concentration in determining the proliferated culture (%) during proliferation stage. M_3 containing BAP (5.0 mgl^{-1}) resulted in significantly highest proliferated cultures (Fig 2). Similarly number of shoots per explant produced showed positive relation with BAP concentration upto a level, after which further increase in BAP concentration inhibited shoot proliferation (Table 4). M_3 medium resulted in maximum number of shoots per explant (2.53) followed by M_2 (2.38) and M_1 (2.14). Maximum number of shoots per explant (2.86) was produced at BAP (2.5 mgl^{-1}) which was significantly higher than 2.60 at BAP (5.0 mgl^{-1}) and 2.50 at BAP (0.5 mgl^{-1}). Interaction effect revealed that there is no significant interaction between media and growth regulator concentration on number of shoots produced per explant. M_2 fortified with BAP (2.5 mgl^{-1}) resulted in maximum number of shoots per explant (3.08) followed by 2.84 in M_3 supplemented with BAP (5.0 mgl^{-1}).

Table 3 Effect of media type and growth regulator level (mgl^{-1}) on proliferated cultures (%)

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
BAP (0.5)	55.47	48.39	66.51	56.79
BAP (2.5)	62.16	59.28	68.48	63.31
BAP (5.0)	65.78	69.62	79.47	71.62
Control	21.01	27.82	12.92	20.58
Mean	51.11	51.28	56.85	
C.D ($p \leq 0.05$)	Media (A) = 2.620, BAP (B) = 3.025, A × B = 5.240			

Table 4 Effect of media type and growth regulator level (mgl^{-1}) on number of shoots/explant

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
BAP (0.5)	2.30	2.46	2.75	2.50
BAP (2.5)	2.69	3.08	2.80	2.86
BAP (5.0)	2.46	2.51	2.84	2.60
Control	1.09	1.47	1.73	1.43
Mean	2.14	2.38	2.53	
C.D ($p \leq 0.05$)	Media (A) = 0.168, BAP (B) = 0.194, A × B = NS			

Table 5 Effect of media type and growth regulator level (mgl^{-1}) on av. length of shoots (mm)

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
BAP (0.5)	43.13	42.48	42.78	42.80
BAP (2.5)	39.55	31.10	42.22	37.62
BAP (5.0)	36.18	20.13	41.38	32.56
Control	43.39	40.40	51.33	45.04
Mean	40.56	33.53	44.43	
C.D ($p \leq 0.05$)	Media (A) = 2.126, BAP (B) = 2.155, A × B = 4.052			

Average length of shoots also varied with type of media and change in growth regulator level (Table 5). There was decrease in average length of shoots with an increase in level of BAP. Maximum shoot length (44.43 mm) was produced in M_3 media which was significantly different and higher as compared to shoot length produced in M_1 (40.56 mm) and M_2 (33.53 mm). Shoots of the most desirable length (45.04 mm) was produced in media having no growth regulator i e control followed by media supplemented with BAP (0.5 mgl^{-1}). Shoot length was also influenced significantly by considering media and growth regulator level in interactive manner. Maximum average shoot length (51.33 mm) was obtained in M_3 medium containing no growth regulator (basal M_3 medium) i e control. It was significantly higher as compared to all treatment combinations. A number of factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explants development stage, etc affect shoot proliferation, shoot length and shoots per explant (Bhat *et al*. 2012). An initial lag period due to slow growth of cultures has been reported in *Pyrus pyrifolia* (Thakur and Kanwar 2008a) and *Pyrus calleryana* (Stimart and Harbage 1989). Dwivedi and Bist (1999) in *P. pyrifolia* cv. Gola and Thankur and Kanwar (2008a) in *P. pashia* also reported superiority of WPM over MS and ½ MS media. However, Ciccotti *et al.* (2008) observed better proliferation in MS medium and higher strength MS medium over WPM medium in *Malus* species and this may be due to different genotype (Webster and Jones 1991). Cytokinins overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. The effective concentration of exogenous cytokinin required to reverse apical dominance varies with the culture systems. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by, in decreasing order, kinetin and 2-iso-pentyladenine (2ip) (Hu and Wang 1983). Similarly a

positive correlation was reported between BA concentration and shoot multiplication up to a certain BA level by Thakur and Kanwar (2008a) in *P. pashia*, Shibli *et al.* (1997) in *P. syrica*, Karimpour *et al.* (2013) in *P. pyrifolia* cv. Sebri. Although increasing BAP concentration in shoot multiplication medium enhanced proliferation rate but shoot length decreased significantly. Increase in shoot proliferation and decrease in shoot length following increase in cytokinin concentration in various media has been reported in *Pyrus* species, Karimpour *et al.* (2013).

Fig 3 Rooting of Patharnakh using M_1 medium containing IBA (1.0 mgl^{-1})

In vitro rooting

The *in vitro* regenerated shoots (>30 mm) obtained during shoot multiplication stage were separated from the shoot clump. These separated shoots were transferred to rooting media i e M_1, M_2 and M_3 containing different growth regulators namely IBA and NAA at different concentrations. Data on the effect of type of media and plant growth regulator on rooting (%), number of roots per explant and average length of roots was calculated. NAA didn't induce any rooting in Patharnakh irrespective of media used. However, very little rooting was obtained by supplementing rooting media with IBA. Use of particular media and level of IBA has certainly an effect on rooting (%) in Patharnakh (Table 6). Maximum rooting of 3.08 percent was obtained on M_1 medium followed by 1.04 percent on M_2 and 0.96 percent on M_3 medium. IBA at 1.0 mgl^{-1} induced maximum rooting (6.40%), which was significantly higher than 2.09 percent obtained at IBA (1.5 mgl^{-1}). The interaction effect of the treatment combination of M_1 and IBA (1.0 mgl^{-1}) resulted in maximum rooting percentage of 9.16 (Fig 3).

Perusal of data further reveals that there is significant effect of type of rooting media and IBA levels on number of roots produced per explants (Table 7). The highest number of roots per explant (0.86) was produced in M_1, which is significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (1.94) was obtained by using IBA at 1.0 mgl^{-1}. M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant (2.60) than what was obtained in M_2 medium fortified with IBA (1.5 mgl^{-1}).

M_1 rooting medium resulted in longer average root length of 7.04 mm than M_3 (6.63 mm) and M_2 (5.04 mm) medium (Table 8). The longest average root length was produced by using IBA at 1.0 mgl^{-1}. The interaction effect of M_3 and IBA (1.0 mgl^{-1}) resulted in maximum average root length of 33.13 mm followed by 25.20 mm in M_2 fortified with IBA (1.0 mgl^{-1}). Patharnakh has been reported more difficult to root by Bhojwani *et al.* (1984). Since various media used for rooting were solidified with agar and thus poor rooting response may be due to poor aeration (Torres 1988). Similarly, Kohlenbach and Wernicke (1978) reported that agar can have a suppressive effect on rooting response of *Pyrus* species. Variation in rooting response among the *Pyrus* genotypes may be due to difference in auxin uptake and metabolism (Beraldi *et al.* 1993). Huettman and Preece (1993) suggested that rooting of microshoots during micropropagation of various plant species proves difficult because of carry over effect of cytokinin used during proliferation stage. Better results in terms of rooting (%) on ½ MS are in conformity with Baviera *et al.* (1989) in Conference pear, Dwivedi and Bist (1999) in Gola pear, Hassanen and Gabr (2012) in *P. betulaefolia*. The promotory effect of a diluted mineral solution on rooting can be explained by the reduction of nitrogen concentration (Al-Bahir *et al.* 1999). Kikas *et al.* (2006), Rai *et al.* (2010) also reported that high salt concentration of MS promoted active proliferation, but the shoots remained too short to induce rooting during rooting stage, but with low salt concentration of MS, the proliferation rate was lower but shoots elongated enough for better rooting. Hu and Wang (1983), Thakur and Kanwar (2008b) reported that when the auxin concentration is too high, callus would form as the shoot base inhibiting normal root development. Variation in rooting response in various media at same auxin level was reported by Novak and Juvova (1983) because the effect of auxins on rooting of shoots depends on the mineral composition of nutrient media. The effect of mineral concentration of the culture medium on rooting can be attributed to the participation of inorganic ions in processes regulating hormonal balance (Amzallag *et al.* 1992).

Table 6 Effect of media type and IBA level (mgl^{-1}) on rooting (%)

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	9.16	5.22	4.82	6.40
IBA (1.5)	6.26	0.00	0.00	2.09
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	3.08	1.04	0.96	
C.D ($p \leq 0.05$)	Media (A) = 0.201, IBA (B) = 0.258, A × B = 0.446			

Table 7 Effect of media type and IBA level (mgl^{-1}) on number of roots/explant

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	2.60	1.80	1.41	1.94
IBA (1.5)	1.68	0.00	0.00	0.56
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	0.86	0.36	0.28	
C.D ($p \leq 0.05$)	Media (A) = 0.034, IBA (B) = 0.438, A × B = 0.758			

Table 8 Effect of media type and IBA level (mgl^{-1}) on average length of roots (mm)

Growth regulator (mgl^{-1})	Media			Mean
	M_1 (½ MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	14.22	25.20	33.13	24.18
IBA (1.5)	20.96	0.00	0.00	6.99
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	7.04	5.04	6.63	
C.D ($p \leq 0.05$)	Media (A) = 0.524, IBA (B) = 0.676, A × B =1.171			

The effect of various media on establishment, proliferation and rooting revealed that lowest necrotic culture percentage was observed by using M_2 medium and maximum establishment was obtained on M_3 medium supplemented. Maximum proliferated cultures (79.47%) were obtained using M_3 medium fortified with BAP (5.0 mgl^{-1}). However, maximum shoots per explant (3.08) were produced in M_2 medium supplemented with BAP (2.5 mgl^{-1}). Shoots of maximum length (53.75 mm) were obtained using M_3 medium containing BAP (0.5 mgl^{-1}). Rooting was maximum (9.16%) using M_1 medium fortified with IBA (1.0 mgl^{-1}) while as no rooting was obtained irrespective of media using NAA. Roots per explant were 2.60 using M_1 medium supplemented with IBA (1.0 mgl^{-1}). However, roots of maximum length were obtained using M_3 medium supplemented with IBA (1.0 mgl^{-1}).

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MICROPROPAGATION OF KAINTH (Pyrus pashia) - AN IMPORTANT ROOTSTOCK OF PEAR IN NORTHERN SUBTROPICAL REGION OF INDIA

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Necrotic culture

ABSTRACT

Micropropagation of Kainth using nodal explants was carried out in tissue culture laboratory, Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-13. The effect of various media {1/2 MS (M_1), MS (M_2) and WPM (M_3)} and growth regulators (BAP, IBA and NAA) on establishment, proliferation and rooting was studied. Per cent necrotic culture was found to be influenced by type of media and growth regulator fortification during establishment stage. Least necrotic culture percentage in Kainth was observed by using M_2 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). M_2 medium containing BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) gave maximum explant establishment (52.80 %). During proliferation stage, M_3 medium supplemented with BAP (3.0 mgl^{-1}) resulted in highest proliferated cultures (83.19%). Similarly the highest shoots per explant were obtained using M_3 medium supplemented with BAP (3.0 mgl^{-1}). However, shoots of maximum length were obtained in M_3 medium containing BAP (0.0 mgl^{-1}) i.e control. Per cent rooting, roots per explant and root length was found to be influenced by type of medium and growth regulator fortification. IBA (0.1 mgl^{-1}) induced maximum rooting (14.08 %) using M_1 medium however, NAA (1.0mgl^{-1}) induced rooting of 27.46 per cent using M_1 medium. Similarly using IBA, maximum roots per explant were obtained using M_1 medium supplemented with IBA (1.0mgl^{-1}). However, NAA (1.0 mgl^{-1}) induced highest roots per explant i.e 3.60 using M_2 medium. M_3 supplemented with IBA (0.1 mgl^{-1}) resulted in maximum root length of 31.10 mm however, NAA (1.0 mgl^{-1}) resulted in maximum root length of 23.22 mm using M_3 medium.

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1 Introduction

Presently pear (Pyrus spps) is next to apple in importance, acreage, production and varietal wealth among temperate fruits in India. It can grow under wider temperature conditions ranging from minus 26^0C when dormant to as high as 45^0C during growth period (Chadha, 2001). Pear is grown from warm humid sub-tropical plains to cold dry temperate regions of India occupying an area of 49340 ha with the annual production of 317270 MT (indiastat, 2013). The area can be increased further and cultivation of this crop may prove to be a best alternative for diversification of agriculture. Seedling rootstocks are not uniform in growth and productivity (Baviera et al., 1989). Therefore, vegetative propagation methods like cutting and stooling are used to multiply pear rootstocks. In vitro propagation has shown promises for rapid and large scale clonal multiplication of disease free planting material throughout the year. In vitro propagation has been reported in several pear rootstocks like P betulaefolia L. (Hassanen & Gabr 2012), wild pear (Thakur & Kanwar 2008), OPR 157, OPR 260 and OH × F 230 (Yeo & Reed 1995), P calleryana (Antunes de et al., 2004), Pyrodwarf (Ruzic et al., 2011) and P communis rootstock (Rahman et al., 2007).

2 Materials and Methods

Kainth plants growing in Fruit Research Farm, Department of Fruit Science, PAU, Ludhiana were used as a source of explants in the present study. Nodal segment explants were taken from current season's growth from March to November. The explant source was subjected to uniform cultural practices. The basal media used in the study were Murashige & Skoog's medium with half strength of macro and micronutrients (M_1), Murashige & Skoog's medium (M_2) and Woody Plant Medium (M_3).

Explants were first washed in running tap water for 15 minutes followed by keeping in 1 per cent bavistin along with few drops of Tween-20 for 20 minutes. Later on explants were washed thoroughly by keeping under running tap water till all residues gets washed out. Before culturing, explants were sterilized with 0.1 per cent $HgCl_2$ for 5 minutes within laminar air flow cabinet, followed by 3-4 washing using sterile water.

The hood of the cabinet was thoroughly cleaned by cotton dipped in methanol. All the instruments used were autoclaved before culturing. The explants were inoculated in test tubes/glass jars containing autoclaved media using sterilized forcep for establishment. Different media (½ MS, MS and WPM) fortified with different combinations of 6-benzylaminopurine (BAP) (0.5-3.0 mgl^{-1}) and IBA (0.01-2.0 mgl^{-1}) were used during establishment stage. The data were recorded on per cent necrotic cultures and explant establishment after 3 weeks of inoculation.

The explants which got established on medium were used as material for proliferation. Necessary dissection of established explants was carried out using sterilized blade and forcep before transfer to shoot proliferation media. Various shoot proliferation media i.e. M_1, M_2 and M_3 fortified with various concentrations of BAP i.e. 0.5-5.0 mgl^{-1} were used. The optimum media and concentration of BAP for shoot proliferation was standardized. Observations on per cent proliferated cultures, number of shoots/explant and average length of shoot (mm) was recorded after third subculture and the culture duration were five weeks each.

After allowing shoots to multiply on shoot proliferation medium, individual shoots were separated (>30mm) and transferred to root regeneration medium. Different types of media i.e. M_1, M_2 and M_3 containing various combinations of IBA (0.1-2.0 mgl^{-1}) and NAA (0.1-2.0 mgl^{-1}) were used. Observations on per cent rooting, number of roots/ explant and average length of roots (mm) were recorded four weeks after culturing. The data generated in course of the present study was analyzed using completely randomized design (factorial), replicated 3 times.

3 Results and Discussion

3.1 Effect of various media supplemented with growth regulators on necrotic cultures

The effect of different media and growth regulator combination on necrotic culture induction in Kainth explants during establishment stage is presented in Table 1. These data reveal exists the significance of the influence of media type and growth regulator concentration on per cent necrosis in Kainth explants during tissue culture. M_2 medium proved best in terms of least necrotic culture induction of 11.32 per-cent, followed by M_1 and M_3 which resulted in 14.79 and 14.98 per cent of necrotic cultures respectively. M_1 and M_3 medium were statistically similar in terms of induction of necrosis. The least per cent necrosis (6.35) was observed in media supplemented with BAP (1.5 mgl^{-1}) and IBA (1.0 mgl^{-1}), it was followed by combination of BAP (3.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) resulting in 9.11 per cent of necrotic cultures. Cumulative effect of media and growth regulator revealed that the lowest necrotic culture (2.10 %) was observed by using M_2 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by M_2 fortified with BAP (3.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum necrotic culture percentage of 28.66 was observed in M_1 medium fortified with BAP (0.5 mgl^{-1}) and IBA (2.0 mgl^{-1}). Almost all treatment combinations differ significantly in terms of induction of necrosis during establishment stage of in vitro culture. Although there is considerable work done on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their in vitro effects (Bairu et al., 2009). But a variation observed in necrosis at various combinations of growth regulators has been credited to medium composition and growing conditions by Bairu et al. (2009).

Table 1 Effect of various media types and growth regulators (mgl^{-1}) on per-cent necrosis.

Growth regulator combination (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	15.48	7.79	8.69	10.65
BAP(0.5)+IBA(1.0)	25.23	22.17	10.35	19.25
BAP(0.5)+IBA(2.0)	28.66	27.52	10.80	22.33
BAP(1.5)+IBA(0.01)	4.71	2.10	12.25	6.35
BAP(1.5)+IBA(1.0)	17.25	7.11	23.17	15.84
BAP(1.5)+IBA(2.0)	15.16	9.48	15.47	13.37
BAP(3.0)+IBA(0.01)	6.62	3.50	17.22	9.11
BAP(3.0)+IBA(1.0)	10.25	4.27	20.16	11.56
BAP(3.0)+IBA(2.0)	8.05	14.33	17.52	13.30
Control	16.45	14.89	14.19	15.18
Mean	14.79	11.32	14.98	
C.D ($p \leq 0.05$)	Media (A)=0.342, GR's (B)=0.706, A×B=1.296			

Equilibrium between phenolic compounds and hormones quite affects the success on plant tissue culture (Poessel et al., 1980). These results are in conformity with earlier observations made by De Paoli (1989) and Leite et al., (1997), who reported Murashige and Skoog medium as the best medium for culture initiation resulting in the highest per cent establishment with least necrotic cultures. Higher necrosis in Woody Plant Medium may be attributed due to lower total nitrogen content (Mamaghani et al., 2010).

3.2 Effect of medium on per cent explant establishment of Kainth

Highest establishment was reported in M_2 medium as shown in table 2. Explant establishment of 32.14 per-cent was achieved in M_2 medium and it was statistically higher as compared to M_1 and M_3 medium. Similarly, higher explant establishment (52.53 %) was reported from the media supplemented by BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}). The interaction effect showed that the treatment combination of M_2 containing BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) gave maximum per-cent of explant establishment (63.60) {Fig 1} whereas; minimum explant establishment of 10.68 per cent was obtained on M_3 supplemented with BAP (0.0 mgl^{-1}) and IBA (0.0 mgl^{-1}) i.e. control. Variations in per cent establishment of explants with different doses of auxin and cytokinin during micropropagation are in conformity with the reports given by Fan & Jiang (1993) in apple; Mondal et al., (1994) in Carica papaya; Caboni et al., (1999) in pear; Chakravarty & Goswami (1999) in Citrus acida; Akbar et al., (2003) in pineapple and Canli & Tian (2008) in sweet cherry. Different media have been tried earlier for establishment of plant species by various workers and reported varied results in terms of establishment percentage. Peer et al., (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir Grossa using Murashige & Skoog medium over Driver & Kuniyuli medium, Woody Plant Medium & Knop's macro and MS micro-organics medium independent of growth regulators concentration. In vitro effects of growth regulator on overall establishment of explant have been reported to be influenced by growth medium composition, growing conditions and genotype (Bairu et al., 2009, Karimpour et al., 2013).

Table 2 Effect of various media types and growth regulators (mg/l) on per cent explant establishment.

Growth regulator combination (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	36.80	23.08	18.25	26.04
BAP(0.5)+IBA(0.25)	28.63	25.11	20.02	24.59
BAP(0.5)+IBA(0.5)	12.54	21.88	18.21	17.54
BAP(1.0)+IBA(0.01)	30.86	36.40	36.34	34.53
BAP(1.0)+IBA(0.25)	40.01	36.37	23.07	33.15
BAP(1.0)+IBA(0.5)	19.98	30.75	38.45	29.73
BAP(1.5)+IBA(0.01)	37.58	50.42	18.60	35.53
BAP(1.5)+IBA(0.25)	46.62	**63.60**	47.38	**52.53**
BAP(1.5)+IBA(0.5)	15.77	14.31	18.68	16.25
Control	13.40	19.45	10.68	14.51
Mean	28.22	**32.14**	24.97	
C.D ($p \leq 0.05$)	Media (A)=1.001, GR's (B)= 1.827, A×B=3.165			

3.3 In vitro shoot proliferation

Data regarding shoot proliferation which comprised of per cent proliferated culture, number of shoots per explant and average length of shoots was obtained after two sub culturing as shoot growth was very slow initially irrespective of media used and growth regulator level. Data of in vitro shoot proliferation has been given in Table 3, and these results clearly depicts that in Kainth, percentage of proliferated cultures was significantly affected by various treatment combinations. Statistically maximum proliferated cultures (59.09 %) were obtained on M_3 medium. With respect to BAP concentration, maximum proliferated cultures (64.79 %) were obtained by using BAP (3.0 mgl^{-1}), irrespective of media used. Similarly interaction values show that maximum proliferated cultures (83.19 %) were obtained by employing M_3 medium supplemented with BAP (3.0 mgl^{-1}) {Figure 2}.

From the perusal of data in Table 4, it is evident that number of shoots per explant is influenced by both treatments i.e. type of media and growth regulator concentration. The highest number of shoots per explant (4.10) was obtained when M_3 was used as shoot proliferation medium. M_1 and M_2 resulted in significantly lesser number of shoots per explants. Data in Table 4 clearly reveals that maximum number of shoots was obtained by using higher dose of BAP. Increasing BAP level from 0.5 mgl^{-1} to 3.0 mgl^{-1} resulted in increase in number of shoots per explant from 2.95 to 4.65. There existed a significant interaction between type of media and various levels of BAP by effecting number of shoots produced per explant. Maximum number of shoots per explant (5.93) was obtained in M_3 medium fortified with BAP (3.0 mgl^{-1}) followed by M_3 supplemented with BAP (1.5 mgl^{-1}).

Data regarding shoot length as influenced in by type of media and various doses of BAP is presented in Table 5. It is clearly evident that shoot length decreased with an increase in BAP dosage although number of shoots per explant increased significantly. Shoots of most desirable length were obtained on M_3 medium, which was significantly higher than on M_2 and M_1 medium. Data in Table 5 reveal that BAP (0.5 mgl^{-1}) is best in terms of production of the most desirable shoot length (41.47 mm), which is at par with control (39.97) containing no BAP. There was a significant interaction between type of media and BAP concentration for average length of shoots produced. Longest shoots (46.32 mm) were obtained in M_3 medium that was free of BAP followed by M_3 fortified with BAP (0.5 mgl^{-1}). These two treatments were significantly at par with respect to each other but were significantly better in terms of producing most desirable shoot length when compared with other treatment combinations. These findings are in conformity with those of Dwivedi & Bist (1999) in P. pyrifolia cv. Gola and Kadota et al. (2001) in Japanese pear cultivar Hosui, who reported superiority of WPM over other media with respect to proliferation rate. BAP level was found to effect significantly per cent proliferation, shoots per explant and shoot length and these results are in conformity with studies reported by Dwivedi & Bist (1999), Sedlak & Paprstein (2003), Cosac & Frasin (2008), Karimpour et al. (2013), Hassanen & Gabr (2012), Ruzic et al. (2011), Isikalan et al. (2011) and Soni et al. (2011). Hu & Wang (1983) reported that cytokinins, especially BAP stimulated axillary bud development but at higher concentration shoot elongation was suppressed. Similarly, higher number of shoots per explant during proliferation stage on M_2 as compared to M_1 has been reported by Hassan (2012) in Le Conte pear, Tange et al. (2008) in Bartlett pear and Mustafa et al. (2013) on fig due to higher nutrient concentration.

Table 3 Effect of media types and growth regulator level (mgl-1) on per cent proliferated cultures.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	63.25	36.63	50.49	50.12
BAP(1.5)	45.12	52.92	73.32	57.12
BAP(3.0)	70.79	40.38	83.19	64.79
Control	11.73	14.35	29.36	18.48
Mean	47.72	36.07	59.09	
C.D ($p \leq 0.05$)	Media (A)= 2.352 , BAP (B)= 2.716, A×B=4.705			

Table 4 Effect of various media types and growth regulator level (mgl^{-1}) on number of shoots/explant.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	2.33	3.70	2.81	2.95
BAP(1.5)	2.90	4.23	5.13	4.09
BAP(3.0)	3.28	4.74	5.93	4.65
Control	1.54	2.23	2.51	2.09
Mean	2.51	3.73	4.10	
C.D ($p \leq 0.05$)	Media (A)= 0.126, BAP (B)= 0.145 , A×B=0.251			

Table 5 Effect of various media types and growth regulator level (mgl^{-1}) on average length of shoots (mm).

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	40.65	40.35	43.40	41.47
BAP(1.5)	37.77	35.18	41.00	37.98
BAP(3.0)	28.25	32.22	36.97	32.48
Control	37.23	36.35	46.32	39.97
Mean	35.98	36.03	41.92	
C.D ($p \leq 0.05$)	Media (A)= 1.582, BAP (B)= 1.826 , A×B=3.163			

Table 6 Effect of various media types and IBA level (mgl^{-1}) on per cent rooting.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	14.08	8.90	8.39	10.46
IBA (1.0)	8.67	0.00	0.00	2.89
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	5.69	2.23	2.10	
C.D($p \leq 0.05$)	Media (A)= 0.814, IBA(B)=0.940, A×B=1.628			

Table 7 Effect of various media types and IBA level (mgl^{-1}) on number of roots per explant.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	1.98	1.86	1.65	1.83
IBA (1.0)	2.60	0.00	0.00	0.87
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	1.15	0.47	0.41	
C.D($p \leq 0.05$)	Media (A)= 0.053, IBA(B)= 0.061, A×B=0.106			

Table 8 Effect of various media types and IBA level (mgl^{-1}) on average length of roots (mm).

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	23.30	29.00	31.10	27.80
IBA (1.0)	19.60	0.00	0.00	6.53
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	10.73	7.25	7.78	
C.D($p \leq 0.05$)	Media (A) = 0.856, IBA (B) = 0.988. A×B=1.711			

3.4 In vitro rooting of Kainth.

The in vitro regenerated shoots during shoot proliferation were transferred to various rooting media supplemented with different levels of IBA and NAA. Data pertaining to the per cent rooting, roots per explant and average length of roots (mm) as affected by type of media and IBA levels is presented in Tables 6, 7 and 8. The in vitro shoots obtained during proliferation stage were transferred to various rooting media supplemented with different levels of IBA (mgl^{-1}). Data of Table 6 clearly reveal that per cent rooting was effected significantly by type of media and level of IBA. The highest level of rooting (5.67 %) was noticed in M_1 medium followed by M_2 (2.23 %) and lowest one reported in M_3 medium (2.10 %). Media supplemented by IBA ($0.1mgl^{-1}$) favour rooting and highest rooting percentage (10.46) was obtained by this combination, which was significantly higher than rooting (%) at higher levels of IBA i.e. 2.89 per cent at IBA ($1.0\ mgl^{-1}$). The highest rooting (14.08 %) was observed in M_1 medium fortified with IBA ($0.1\ mgl^{-1}$) followed by 8.90 per cent in M_2 supplemented with IBA ($0.1\ mgl^{-1}$) {Figure 3}.

Figure 1 Establishment of Kainth using M_2 fortified with BAP (1.5mgl^{-1}) and IBA (0.25mgl^{-1})

Figure 2 Proliferation of Kainth using M_3 fortified with BAP (3.0mgl^{-1})

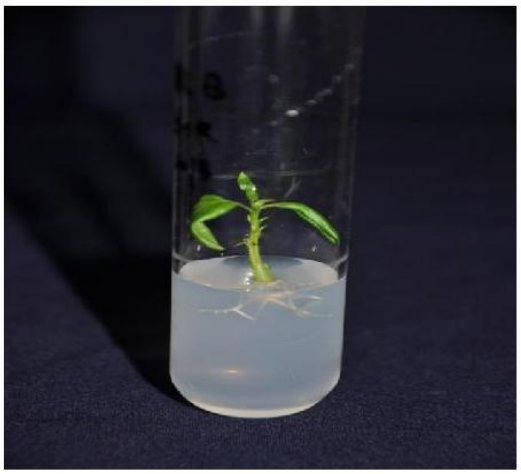

Figure 3 Rooting in Kainth in M_1 containing IBA (0.1mgl^{-1})

Figure 4 Rooting in Kainth using M_1 fortified with NAA (1.0mgl^{-1})

Plate I Establishment, proliferation and rooting of Kainth.

Data in Table 7 represents the effect of rooting media and IBA levels (mgl^{-1}) on number of roots per explant. The highest number of roots per explant (1.15) was produced in M_1, which is significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (1.83) was obtained by using IBA at 0.1 mgl^{-1} irrespective of media used. M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant (2.60) than rest of treatment combinations.Roots of maximum length (10.73 mm) were obtained in M_1 medium which is significantly higher than root length achieved in M_3 and M_2 medium (Table 8). Irrespective of media used, IBA (0.1 mgl^{-1}) resulted in longer roots (27.80 mm) than root length obtained at IBA (1.0 mgl-1).

Interaction effect revealed that roots of maximum length (31.10 mm) were produced in M_3 medium fortified with IBA (0.1 mgl^{-1}), which is significantly higher than other treatment combinations.

Tables 9, 10 and 11 shows the data regarding the effect of type of rooting media and various concentrations of NAA levels on rooting per cent, number of roots per explant and average length of roots (mm) respectively. Data in Table 9 clearly reveal per cent rooting induced by NAA was higher than rooting induced by IBA and rooting was effected significantly by type of media and level of NAA. The highest level of rooting (12.65 %) was noticed in M_1 medium followed by 11.83 per cent in M_2 and lowest (9.57 %) in M_3 medium. Rooting percentage of 22.90 was obtained using NAA (1.0mgl^{-1}) irrespective of type of rooting media, which is significantly at par to rooting obtained at NAA (0.1 mgl^{-1}) i.e. 22.51 %. The highest rooting (27.46 %) was observed in M_1 medium fortified with NAA (1.0 mgl^{-1}) {Figure 4} followed by 24.58

per cent in M_2 medium supplemented with NAA (1.0mgl^{-1}).Number of roots per explant was significantly higher by using M_2 medium irrespective of NAA fortification (Table 10). NAA at 1.0 mgl^{-1} induced maximum number of roots per explant (2.84) followed by 2.63 using NAA (0.1 mgl^{-1}). An interaction between various media and NAA concentration on roots per explant was significant resulting in 3.60 roots per explant on M_2 medium supplemented with NAA (1.0 mgl^{-1}) followed by 3.26 roots per explant in M_1 fortified with NAA (1.0 mgl^{-1}). Similarly, root length was seen to be significantly affected by type of media and NAA level (Table 11). Statistically longer roots (8.32 mm) were observed on M_1 medium followed by M_2 and M_3, irrespective of NAA dosage. NAA at 0.1mgl^{-1} resulted in longer average root length of 19.95 mm. Interaction values of average root length (mm) indicate that type of media and NAA levels has significant effect on this parameter. M_3 medium supplemented with NAA (1.0mgl^{-1}) resulted in longer roots (23.22 mm) followed by 21.56 mm in M_3 fortified with NAA (0.1mgl^{-1}). Thakur (2004) and Thakur & Kanwar (2008) also reported better rooting response of Kainth as compared to scion varieties. Like the multiplication rate, rooting ability being genotype dependent (Sharma et al. 2007) and rootstocks usually root with greater ability than scions (Dobranszky & Teixeira da Silva, 2010). The mineral concentration in the culture medium affects rooting characteristic and some researchers have proposed that reduction of salt strength to half strength improved rooting (Dimassi-Theriou & Economou, 1993). The reason behind increasing rooting rate on half strength culture medium might be due to a disorder in carbohydrate to nitrogen in nutrient medium, which lead to decreasing nitrogen level in shoot and then improving rooting rate, initiation roots, increasing root number and lengths (Fotopoulos & Sotiropoulos 2005). Higher rooting response in Kainth by using NAA as compare IBA are in conformity with Thakur (2004) and Thakur & Kanwar (2008). Better rooting response of pear genotypes with NAA is in concordance to the findings of Singha (1980) who preferred NAA over IBA for inducing roots in P. communis cv. Seckel to avoid the basal callus formation. Reed (1995) also found that some pear genotypes rooted better on NAA than on IBA. Too high an auxin concentration in rooting media is undesirable as it leads reduction in rooting by inducing basal callus formation (Lane 1979) or by inhibiting the root elongation (Thimann 1977). This may be the reason for poor rooting response at higher auxin concentration in the present study.

Table 9 Effect of various media types and NAA level (mgl^{-1}) on per-cent rooting.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	**M_2(MS)**	**M_3(WPM)**	
NAA (0.1)	23.15	22.74	21.63	22.51
NAA (1.0)	27.46	24.58	16.65	22.90
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	12.65	11.83	9.57	
C.D($p \leq 0.05$)	**Media (A)=0.754, NAA(B)=0.871, A×B=1.508**			

Table 10 Effect of various media types and NAA level (mgl^{-1}) on number of roots per explant.

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	**M_2(MS)**	**M_3(WPM)**	
NAA (0.1)	2.98	3.02	1.90	2.63
NAA (1.0)	3.26	3.60	1.66	2.84
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	1.56	1.66	0.89	
C.D($p \leq 0.05$)	**Media (A)= 0.061, NAA(B)= 0.070, A×B=0.121**			

Table 11 Effect of various media types and NAA level (mgl^{-1}) on average length of roots (mm).

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2 MS)	**M_2(MS)**	**M_3(WPM)**	
NAA (0.1)	18.97	19.33	21.56	19.95
NAA (1.0)	14.30	14.22	23.22	17.25
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	8.32	8.39	11.20	
C.D($p \leq 0.05$)	**Media (A)= 0.705, NAA(B)= 0.814, A×B=1.409**			

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Research Paper

MICROPROPAGATION OF PATHARNAKH (PYRUS PYRIFOLIA (BURM F.) NAKAI) PEAR USING EXPLANTS OBTAINED FROM FORCED CUTTINGS

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Nodal explants from forced cuttings of Patharnakh were used for micropropagation protocol development. The effect of different media {1/2 MS (M_1), MS (M_2) and WPM (M_3)} and growth regulators (BAP, IBA and NAA) on establishment, proliferation and rooting was examined. Necrotic culture (%) was found to be influenced by type of media and growth regulator fortification during establishment stage. Lowest necrotic cultures (0.00%) were observed in M_2 medium fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum establishment (96.29%) was obtained on M_2 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum proliferated cultures (85.67%) were obtained using M3 medium fortified with BAP (2.5 mgl^{-1}). Similarly the highest shoots per explant (3.80) were obtained in M_3 medium supplemented with BAP (2.5 mgl^{-1}). Shoots of maximum length (53.75) were obtained in M_3 medium containing BAP (0.0 mgl^{-1}), i.e., control. Rooting (%) was maximum (10.16%) using M_1 medium fortified with IBA (1.0 mgl^{-1}). No rooting was obtained irrespective of media using NAA. Roots per explant were 2.38 using M_1 medium supplemented with IBA (1.0 mgl^{-1}). However, roots of maximum length were obtained using M_3 medium supplemented with IBA (1.0 mgl^{-1}).

Keywords: Nodal explants, Micropropagation, Establishment, Proliferation, Necrotic cultures, Rooting

INTRODUCTION

Commercial pear production is mainly represented by *Pyrus* communis (European pear), *Pyrus pyrifolia* (Asian or Oriental pear) and their hybrids. Most of the cultivars belonging to Pyrus communis are suitable for cultivation in temperate climate; however, the Pyrus pyrifolia cultivars are well adapted to sub-tropical climate of north-western states of India. Temperate pear can grow from foothills to high hills (600-2700 m amsl) experiencing 500-1500 chilling hours, while the sub-tropical pears require only 200-300 chilling hours. Pear has become an important fruit crop of Punjab particularly due to recommendation of

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semi-soft pear varieties by PAU. Current pear production relies on Patharnakh (hard pear) which is the leading cultivar of Punjab followed by Baggugosha and Leconte. But recently, semi-soft varieties like Punjab Beauty, Punjab Nectar, Punjab Gold and soft varieties like YaLi and Nijjisseiki have been recommended for general cultivation.

Since, the fruiting characteristics of plants produced from seeds are nearly always inferior to the parent plant due to heterozygosity, thus their seed progeny is not true to type (Jackson and Looney, 1999). However, in vitro propagation gives rise to plants which are genetically identical to the parent plant. In vitro propagation has shown promises for rapid and large scale clonal multiplication of disease free planting plant throughout the year.

MATERIALS AND METHODS

The present investigation entitled, "Micropropagation of Patharnakh (*Pyrus pyrifolia* (*Burm f.*) Nakai) pear using explants obtained from forced cuttings" was carried out in Tissue culture laboratory in the Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-2013. For forcing, dormant cutting (terminal and sub-terminal) of Patharnakh of 15-20 cm in length (10-15 mm diameter) were collected and stored at 4 ±3 °C in polythene bags. After subjecting the requisite chilling units, the cuttings were withdrawn and basal ends were re-cut by about 1 cm and placed in glass jars containing sterile distilled water, covering about 5 cm of basal portion of cuttings. The cuttings were incubated in growth chamber at 23 ± 1 °C under 16 hours photoperiod with light intensity of 3000 lux. The water in glass jars was changed every 4-5 days. Shoots put forth by the sprouted buds served as explant source for micropropagation (Figure 1). Shoots for obtaining explants became ready for first harvesting within 25 ± 5 days. After harvesting first crop of forced explants, the cuttings were again ready for harvesting of second crop within 10 ± 5 days.

The basal media used in the study were Murashige and Skoog's medium with half strength of macro and micronutrients (M_1), Murashige and Skoog's medium (M_2) and Woody Plant Medium (M3). Already prepared Murashige and Skoog's medium (PT021) and Woody Plant Medium (PT026) purchased from HiMedia Pvt. Limited in the form of powder was used to prepare basal media, i.e., M_1, M_2 and M_3. Prescribed amount of powder was dissolved in required amount of distilled water and fortified with measured quantity of growth regulators.

Explants were first washed in running tap water for 15 minutes followed by keeping in 1% bavistin along with few drops of Tween-20 for 20 minutes. Later on explants were washed thoroughly by keeping under running tap water till all residues gets washed out. Before culturing, explants were sterilized with 0.1% $HgCl_2$ for 4 minutes within laminar air flow cabinet, followed by 3-4 washing using sterile water. All the instruments used were autoclaved using newspaper covering before culturing. Surface sterilized explants were transferred to sterilized petridish using sterilized forcep.

The explants were inoculated in test tubes/ glass jars containing autoclaved media using sterilized forcep for establishment. Different media (M_1, M_2 and M_3) fortified with different combinations of 6-benzylaminopurine (BAP) {0.5-1.5 mgl^{-1}} and indolebutyric acid (IBA) {0.01-0.5 mgl^{-1}} were used. After inoculation, culture vessels were incubated at 25 ± 2 °C

Figure 1: Forced Cutting of Patharnakh

temperatures in 16 hours continuous fluorescent light followed by an 8 hours dark period. The incubation temperature and photoperiod was similar in all the experiments conducted. The data were recorded on necrotic cultures (%), explant establishment (%) after 3 weeks of inoculation.

The explants which got established on establishment medium were used as material for proliferation. Necessary dissection of established explants was carried out using sterilized blade and forcep before transfer to shoot proliferation media. Various shoot proliferation media were used, fortified with various concentrations of BAP i.e. 0.5-3.0 mgl^{-1}. The optimum media and concentration of BAP for shoot proliferation was standardized. Observations on proliferated cultures (%), number of shoots/explant and average length of shoot (mm) was recorded after third subculture and the culture duration were five weeks each.

After allowing shoots to multiply on shoot proliferation medium, individual shoots were separated (2 mm) and transferred to root regeneration medium. Different types of media containing various combinations of IBA (0.5-2.0 mgl^{-1}) and NAA (0.5-2.0 mgl^{-1}) were used. Observations on rooting (%), number of roots/ explant and average length of roots (mm) were recorded four weeks after culturing.

RESULTS AND DISCUSSION

The results of present investigation are described under appropriate heads supplemented with tables and figures.

Effect of Medium Supplemented with Growth Regulators on Necrotic Cultures

Data in Table 1 clearly shows that least necrotic culture (4.16%) was observed on M_2 medium. All media differ significantly with respect to necrotic culture induction during establishment. The lowest necrotic culture (4.35%) was observed when BAP (1.0 mgl^{-1}) along with IBA (0.01 mgl^{-1}) was used. Treatment combination of BAP (1.0 mgl^{-1}) and IBA (0.25 mgl^{-1}) was statistically at par when compared with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Significantly higher necrotic culture (%) was observed when no growth regulator was used, i.e., under control. Interaction effect between media and growth regulator revealed that the lowest necrotic culture (%) was observed by using M_2 medium fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by M_2 fortified with BAP (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) and M_3 supplemented with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). These three treatment combinations were at par but differ significantly with rest of treatment combination for induction of necrotic cultures. These results are in conformity with earlier observations made by Liete *et al.* (1997) on Bartlett pear, Singha (1980) on Seckel pear, who reported Murashige and Skoog medium as the best medium for culture initiation resulting in the highest establishment (%) with least necrotic cultures. Higher necrosis in woody plant medium

Table 1: Effect of Media Type and Growth Regulators (mgl^{-1}) on Percent Necrotic Cultures

Growth Regulator Combinations (mgl^{-1})	Media			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	9.02	6.10	9.44	8.19
BAP(0.5) + IBA(0.25)	4.29	3.25	14.18	7.24
BAP(0.5) + IBA(0.5)	8.99	0.57	19.72	9.76
BAP(1.0) + IBA(0.01)	12.38	0.00	0.67	4.35
BAP(1.0) + IBA(0.25)	3.54	3.02	8.38	4.98
BAP(1.0) + IBA(0.5)	5.33	4.53	8.49	6.12
BAP(1.5) + IBA(0.01)	7.22	4.94	8.55	6.90
BAP(1.5) + IBA(0.25)	9.04	4.24	9.30	7.53
BAP(1.5) + IBA(0.5)	12.49	6.79	3.84	7.71
Control	16.83	8.12	14.33	13.09
Mean	8.91	4.16	9.69	
C.D ($p \leq 0.05$)	Media (A) = 0.464, GR's (B) = 0.930, A × B = 1.483			

may be attributed due to lower total nitrogen content (Mamaghani *et al.*, 2010). With regard to growth regulators, various scientists have reported different combinations but variation observed under present study may be due to genotypic difference (Karimpour *et al.*, 2013). Despite considerable research on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their in vitro effects (Bairu *et al.*, 2009). Influence of plant growth regulators on explant necrosis might be influenced by medium composition, growing conditions and genotype (Bairu *et al.*, 2009).

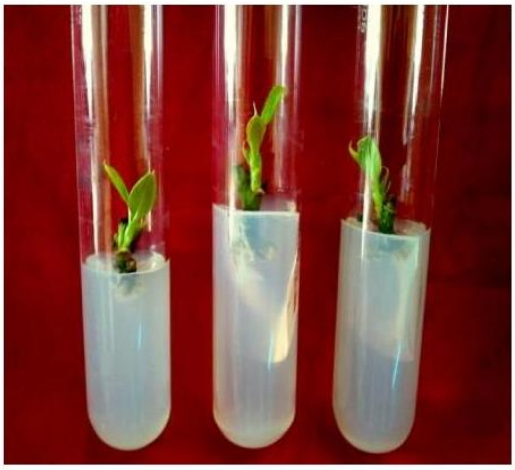
Effect of Medium Supplemented with Growth Regulators on Explant Establishment

Data in Table 2 reveals that both media and growth regulator had significant effect on establishment. Highest explant establishment of 69.68% was obtained using M_2 medium followed by 67.79% on M_1 medium while as BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved the best combination of growth regulators and resulted in the highest explant establishment of 83.57% and it was significantly higher than all other combinations. The interaction effect showed that highest explant establishment of 96.10% (Figure 2) was obtained in M_3 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) and it was at par with M_2 supplemented with BAP (1.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) and M_2 with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). Lowest explant establishment was obtained on M_3 supplemented with BAP (0.0 mgl^{-1}) and IBA (0.0 mgl^{-1}), i.e., under control and it was significantly lower from all treatments. Difference in establishment (%) in various media at different combinations of BAP and IBA may be attributed to varying callusing rates and contaminations values as reported by Kozlina and Jelaska (1987). Different media have been tried earlier for establishment of various plant species by various workers and reported varied results in terms of establishment percentage. Peer *et al.* (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir

Table 2: Effect of Media Type and Growth Regulators (mg/ l) on Percent Establishment (%)

Growth Regulator Combinations (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5) + IBA(0.01)	72.43	62.57	40.20	58.40
BAP(0.5) + IBA(0.25)	80.79	87.56	40.10	69.48
BAP(0.5) + IBA(0.5)	58.74	52.19	54.61	55.18
BAP(1.0) + IBA(0.01)	59.87	70.03	62.08	63.99
BAP(1.0) + IBA(0.25)	84.23	67.99	73.52	75.25
BAP(1.0) + IBA(0.5)	78.51	47.78	52.55	59.61
BAP(1.5) + IBA(0.01)	59.51	95.39	96.10	83.67
BAP(1.5) + IBA(0.25)	88.20	80.26	66.31	78.26
BAP(1.5) + IBA(0.5)	63.80	95.52	63.60	74.31
Control	31.86	37.53	25.58	31.66
Mean	67.79	69.68	57.47	
C.D ($p \leq 0.05$)	Media (A) = 0.996, GR's (B) = 1.818, A × B = 3.150			

Figure 2: Explant Establishment on MS Medium Containing BAP (1.5 mgl^{-1}) + IBA (0.01 mgl^{-1})



Grossa using Murashige and Skoog medium over Driver and Kuniyuli medium, Woody Plant Medium and Knop's macro and MS micro-organics medium independent of growth regulators concentration. Similar results were obtained by Erbenova *et al.* (2001), Dai-Hong *et al.* (2004), Sedlak *et al.* (2008), Hassanen and Gabr (2012), Iglesias *et al.* (2004), Thakur and Kanwar (2008) and Karimpour *et al.* (2013). However variations in establishment (%) observed in terms of growth regulator combination from earlier studies might be due to different genotype, growing conditions, physiological state and cytokinin to auxin ratio of explant (Karimpour *et al.*, 2013). Rathore *et al.* (1991) also reported higher establishment of explants in M_2 medium (MS) supplemented by low level of auxin (IBA) and comparatively higher levels of cytokinin (BAP) in various forest trees.

In Vitro Shoot Proliferation

Shoots from established explants were cultured on different media supplemented with varying doses of BAP. Initially, the shoot growth was very slow in all media at all levels of BAP. However, after two subcultures on respective media, two to three axillary shoots arose from the base of cultured shoots.

As evident from Table 3, percent proliferated cultures were significantly affected by type of

Table 3: Effect of Media Type and Growth Regulator Level (mgl^{-1}) on Percent Proliferated Cultures

Growth Regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	46.30	66.57	68.68	60.52
BAP(2.5)	49.71	54.34	85.67	63.24
BAP(5.0)	60.00	71.72	58.38	63.37
Control	22.56	26.50	40.03	29.70
Mean	44.64	54.78	63.19	
C.D ($p \leq 0.05$)	Media (A) = 9.854 , BAP (B) = 11.378, A × B = NS			

media and BAP concentrations. The highest proliferated culture percent (63.19%) was observed in M_3 followed by 54.78% in M_2, irrespective of BAP concentration. These two media were significantly at par in terms of proliferated cultures. Percent proliferated culture increased with increase in BAP concentration, non-significantly irrespective of type of medium. There was non-significant interaction between type of media and BAP concentration, although the highest per cent proliferated cultures (Figure 3) was obtained in M_3 medium fortified with BAP (2.5 mgl^{-1}).

Figure 3: Proliferation on WPM Containing BAP (2.5 mgl^{-1})

Data in Table 4 show that in all media used, there was considerable increase in number of shoots per explant with increase in BAP concentration upto a level, but further increase in BAP concentration inhibited shoot proliferation significantly in Patharnakh. The highest number of shoots per explant were obtained on M_3 medium (2.85) followed by M_1 (2.67) and M_2 (2.60). M_1 and M_2 were significantly at par in terms of number of shoots per explant production. Maximum number of shoots per explant was obtained at BAP (2.5 mgl^{-1}) which was significantly higher than at BAP (0.5 mgl^{-1}) and BAP (5.0 mgl^{-1}). There was significant interaction between various media and growth regulator concentration for number of shoots per explant produced in Patharnakh. Maximum number of shoots per explant (3.80) was produced in M_3 supplemented with BAP (2.5 mgl^{-1}) followed by 3.66 in M_1 supplemented with BAP (2.5 mgl^{-1}).

Shoot length (mm) was also significantly influenced by type of media and growth regulator levels during proliferation stage of *in vitro* propagation. From the perusal of Table 5, it is evident that maximum average length of shoots (49.71 mm) was obtained in M_3 medium, which was significantly higher than 46.72 mm in M_1 and 36.83 mm in M_2 medium. Maximum length of

Table 4: Effect of Media Type and Growth Regulator Level (mgl^{-1}) on Number of Shoots/ Explant

Growth Regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	3.00	2.77	3.26	3.0
BAP(2.5)	3.66	3.22	3.80	3.6
BAP(5.0)	2.83	3.00	2.67	2.8
Control	1.20	1.41	1.65	1.4
Mean	2.67	2.60	2.85	
C.D ($p \leq 0.05$)	Media (A) = 0.160 , BAP(B) = 0.185, A × B = 0.320			

Table 5: Effect of Media Type and Growth Regulator Level (mgl^{-1}) on Average Length of Shoots (mm)

Growth Regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	50.05	44.53	53.75	49.44
BAP(2.5)	46.20	33.58	49.85	43.21
BAP(5.0)	40.43	25.30	46.33	37.35
Control	50.18	43.91	48.92	47.67
Mean	46.72	36.83	49.71	
C.D ($p \leq 0.05$)	Media (A) = 1.992, BAP (B) = 2.301, A × B = 3.984			

shoots (49.44 mm) were produced at BAP (0.5 mgl^{-1}) followed by 47.67 in control. These two treatments were at par but were significantly higher as compared to BAP (0.5 mgl^{-1}) and BAP (5.0 mgl^{-1}). Interaction studies between media and growth regulator revealed that there was significant interaction between them. M_3 medium supplemented with BAP (0.5 mgl^{-1}) resulted in maximum average length of shoots (53.75 mm) followed by M_1 containing no growth regulator and M_1 fortified with BAP (0.5 mgl^{-1}).

A number of factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explants development stage, etc., affect shoot proliferation, shoot length and shoots per explant (Bhat *et al.*, 2012). An initial lag period due to slow growth of cultures has been reported in *Pyrus pyrifolia* (Bhojwani *et al.*, 1984; and Thakur, 2004) and P calleryana (Stimart and Harbage, 1989). Dwivedi and Bist (1999) in *P. pyrifolia* cv. Gola and Thankur and Kanwar (2008) in *P. pashia* also reported superiority of WPM over MS and ½ MS media. Thakur (2004) reported that in various species of pear, woody plant medium resulted in the highest shoot proliferation rate; however, it was at par with Murashige and Skoog medium. However, Ciccotti *et al.* (2008) observed better proliferation in MS medium and higher strength MS medium over WPM medium in Malus species and this may be due to different genotype (Webster and Jones, 1991). Cytokinins overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. The effective concentration of exogenous cytokinin required to reverse apical dominance varies with

the culture systems. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by, in decreasing order, kinetin and 2-iso-pentyladenine (2ip) (Hu and Wang, 1983). Similarly a positive correlation was reported between BA concentration and shoot multiplication upto a certain BA level by Dwivedi and Bist (1999) in *P. pyrifolia*, Baviera *et al.* (1989) in *P. communis*, Thakur and Kanwar (2008) in *P. pashia*, Karimpour *et al.* (2013) in *P. pyrifolia* cv. Sebri, Ahmad *et al.* (2003) in peach rootstock GF 677 and Silva *et al.* (2003) in *Prunus* 'Capdeboscq' rootstocks. Although increasing BAP concentration in shoot multiplication medium enhanced proliferation rate in Patharnakh but shoot length decreased significantly. Increase in shoot proliferation and decrease in shoot length following increase in cytokinin concentration in various media has been reported in *Pyrus* species by Bhojwani *et al.* (1984), Stimart and Harbage (1989), Berardi *et al.* (1993), Dwivedi and Bist (1999), Thakur and Kanwar (2008) and Karimpour *et al.* (2013). Stimart and Harbage (1989) and Thakur (2004) reported that shoots of desirable length of *P. pyrifolia* can be obtained by lowering cytokinin level in shoot proliferation medium. The optimal BA concentration for maximal shoot proliferation depends on the cultivar (Lane and McDougald, 1982).

In Vitro Rooting

The *in vitro* regenerated shoots (> 30 mm) obtained during shoot proliferation stage were separated from the shoot clump. These separated shoots were transferred to rooting media, i.e., M_1, M_2 and M_3 containing different growth regulators namely IBA and NAA at different concentrations. Data in Table 6 reveal that type of media and growth regulator concentration namely IBA had a significant effect on rooting (%). In general, better rooting was observed in M_1 having rooting (%) of 3.55 followed by 1.70% in M_2 and 1.15% in M_3. All media differ significantly in inducing rooting. Significantly higher rooting percentage of 8.13 was obtained at IBA (1.0 mgl^{-1}) than at IBA (1.5 mgl^{-1}). Rooting was not obtained at either higher levels of IBA ($\leq$ 2 mgl^{-1}) or IBA (<1.0 mgl^{-1}). An interaction between type of media and IBA concentrations on rooting (%) was significant. The highest rooting response (10.16%) was obtained in M1 fortified with 1.0 mgl^{-1} IBA (Figure 4).

The data in Table 7 shows that, irrespective of growth regulator concentrations, statistically higher number of roots were obtained in M_1 (0.80) than in M_2 (0.28) and M_3 (0.24). An average of 1.66 roots per explant was obtained at IBA (1.0 mgl^{-1}), which was statistically higher than 0.53 roots per explant obtained at IBA (1.5 mgl^{-1}). An interaction between various media and IBA concentration on roots per explant was significant resulting in 2.38 roots per explant on M_1 medium supplemented with IBA (1.0 mgl^{-1}).

Figure 4: Rooting on ½ MS Containing I BA (1.0 mgl^{-1})

Table 6: Effect of Media Type and IBA Level (mgl^{-1}) on Percent Rooting				
Growth Regulator (mgl^{-1})	Media			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	10.16	8.48	5.74	8.13
IBA (1.5)	7.58	0.00	0.00	2.53
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	3.55	1.70	1.15	
C.D ($p \leq 0.05$)	Media (A) = 0.302, IBA(B) = 0.389, A × B = 0.674			

Table 7: Effect of Media Type and IBA Level (mgl^{-1}) on Number of Roots per Explant				
Growth Regulator (mgl^{-1})	Media			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	2.38	1.40	1.19	1.66
IBA (1.5)	1.60	0.00	0.00	0.53
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	0.80	0.28	0.24	
C.D ($p \leq 0.05$)	Media (A) = 0.052, IBA(B) = 0.067, A × B = 0.116			

Table 8: Effect of Media Type and IBA Level (mgl^{-1}) on Average Length of Roots (mm)				
Growth Regulator (mgl^{-1})	Media			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	15.90	24.00	28.57	22.82
IBA (1.5)	22.07	0.00	0.00	7.36
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	7.59	4.80	5.71	
C.D ($p \leq 0.05$)	Media (A) = 0.464, IBA(B) = 0.598, A × B = 1.037			

Table 8 represents the data on average length of root (mm) as affected by change in rooting media and various levels of IBA (mgl^{-1}). Overall roots of maximum average length (7.59 mm) were produced in M_1 medium, which was statistically higher as compared to M_3 (5.71 mm) and M_2 (4.80 mm) medium. An average root length of 22.82 mm was produced irrespective of media when IBA (1.0 mgl^{-1}) was used. Interaction effect revealed that roots of maximum length were

produced in M_3 medium fortified with IBA (1.0 mgl^{-1}) having average length of 28.57 mm, which was significantly higher than other treatment combinations. No rooting was observed irrespective of media at various levels of NAA (Data not shown).

Patharnakh has been reported more difficult to root by Bhojwani *et al.* (1984) and Thakur (2004). Reed (1995) screened 49 *Pyrus* genotypes for in vitro rooting response and found it to be genotype dependent. Out of the 49 genotypes, eight failed to root in all the experiments, this included *P. pyrifolia* and *P. pashia*. Since various media used for rooting were solidified with agar and thus poor rooting response may be due to poor aeration (Torres, 1988). Variation in rooting response among the Pyrus genotypes may be due to difference in auxin uptake and metabolism (Berardi *et al.*, 1993). Similarly Kohlenbach and Wernicke (1978) and Lane (1979b) reported that agar can have a suppressive effect on rooting response of Pyrus species. Better results in terms of rooting (%) on ½ MS are in conformity with Marino (1984) in Bartlett pear, Baviera *et al.* (1989) in Conference pear, Bartish *et al.* (1994) in Pyrus communis seedling, Thakur and Kanwar (2008) in various Pyrus species, Dwivedi and Bist (1999) in Gola pear and Hassanen and Gabr (2012) in *P. betulaefolia*. NAA was found to induce higher rooting (%) at lower concentrations than IBA accept in Patharnakh (forced and active). Singha (1980) reported that NAA avoid the basal callus formation and preferred NAA over IBA for inducing roots in *P. communis* cv Seckel. Zimmerman (1983) and Rai *et al.* (2010) reported that for rooting, concentration of mineral salts and sugars should be reduced to half or less of the concentration used for proliferation. Auxin is essential for root initiation and NAA is the most effective auxin for induction of root regeneration. Therefore, NAA is mostly used for inducing rooting followed by IBA, IAA and 2, 4-D (Hu and Wang, 1983). Lane (1979a) observed that high concentration of auxin induce callus at the shoot base which inhibits normal root development. Thimann (1977) and Thakur and Kanwar (2008) reported that high auxin is undesirable for root elongation phase and hence inhibited by high concentration of auxin.

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3.4 *IN VITRO* PROPAGATION OF KAINTH (*PYRUS PASHIA*) USING EXPLANTS FROM FORCED CUTTING

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Abstract

This study was carried out in Tissue culture laboratory, Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-13. The effect of various media {1/2 MS (M_1), MS (M_2) and WPM (M_3)} and growth regulators (BAP, IBA and NAA) on establishment, proliferation and rooting was studied. Necrotic culture (%) was found to be influenced by type of media and growth regulator fortification during establishment stage. M_2 medium supplemented with BAP (1.5mgl^{-1}) and IBA (0.01mgl^{-1}) proved best by inducing least necrotic cultures. However, maximum establishment (63.60 %) was obtained on M_2 containing BAP (1.5mgl^{-1}) and IBA (0.25mgl^{-1}). Maximum proliferated cultures (95.30%) and shoots per explant were obtained using M_3 medium fortified with BAP (3.0mgl^{-1}). However, shoots of maximum length (42.97mm) were obtained in M_3 medium containing BAP (0.0mgl^{-1}) i.e control. Rooting (%), roots per explant and root length was found to be influenced by type of medium and growth regulator fortification. Rooting (%) was maximum (13.34%) was observed in M_1 medium supplemented with IBA (0.1mgl^{-1}). However, NAA (1.0mgl^{-1}) induced rooting of 29.61 per cent using M_1 medium. Maximum roots per explant were obtained using M_1 medium supplemented with IBA (1.0mgl^{-1}). NAA (1.0mgl^{-1}) induced highest roots per explant i.e 3.40 using M_1 medium. However, M_3 supplemented with IBA (0.1mgl^{-1}) resulted in maximum root length of 31.15 mm. NAA (0.1mgl^{-1}) resulted in maximum root length of 22.97 mm using M_1 medium.

Keywords: Establishment, proliferation, rooting, fortification, necrotic cultures

Introduction

The use of tissue culture for fruit and nut tree species have increased substantially since the early 1970s and virtually all fruit tree species have been micropropagated with various degrees of success. Seedling rootstocks are not uniform in growth and productivity (Baviera *et al* 1989). Therefore, vegetative propagation methods like cutting and stooling are used to multiply pear rootstocks. Micropropagation has shown promises for rapid and large scale clonal multiplication

of disease free planting material throughout the year. *In vitro* propagation has been reported in several pear rootstocks *viz. P betulaefolia* L. (Hassanen and Gabr 2012), Wild pear (Thakur and Kanwar 2008); OPR 157, OPR 260 and OH × F 230 (Yeo and Reed 1995); *P calleryana* (Antunes de *et al* 2004) and quince (Dolcet-Sanjuan *et al* 1990), Pyrodwarf (Ruzic *et al* 2011) and *Pyrus communis* L. rootstock (Rahman *et al* 2007).

Materials and Methods

For forcing, dormant cuttings of Kainth of 15-20 cm in length (10-15 mm diameter) were collected and stored at 4±3°C in polythene bags. After subjecting the requisite chilling units, the cuttings were withdrawn and basal ends were re-cut by about 1 cm and placed in glass jars containing sterile distilled water, covering about 5 cm of basal portion of cuttings. The cuttings were incubated in growth chamber at 23±1°C under 16 hours photoperiod with light intensity of 3000 lux. The water in glass jars was changed every 4-5 days. Shoots put forth by the sprouted buds served as explant source for *in vitro* propagation (Fig 1).

The basal media used in the study were Murashige and Skoog's medium (MS), Murashige and Skoog's medium with half strength of macro and micronutrients (1/2 MS) and Woody Plant Medium (WPM). Already prepared MS medium (PT021) and WPM (PT026) purchased from Hi Media Pvt. Limited was used to prepare basal media i.e. M_1 (half strength MS), M_2 (full strength MS) and M_3 (Woody Plant Medium). Sterilized explants were inoculated in test tubes/glass jars containing autoclaved media for establishment. Different media (½ MS, MS and WPM) fortified with different combinations of 6-benzylaminopurine (BAP) {(0.5-3.0mgl^{-1}) and IBA (0.01-2.0 mgl^{-1})} were used. The data were recorded on necrotic cultures (%), explant establishment (%) after 3 weeks of inoculation.

Established explants were transferred to shoot proliferation media. Various shoot proliferation media i.e. ½ MS, MS and WPM were used, fortified with various concentrations of BAP i.e. 0.5-5.0 mgl^{-1}. The optimum media and concentration of BAP for shoot proliferation was standardized. Observations on proliferated cultures (%), number of shoots/explant and average length of shoot (mm) was recorded after third subculture and the culture duration were four weeks each.

After allowing shoots to multiply on shoot proliferation medium, individual shoots were separated (20mm long) and transferred to root regeneration medium. Different types of media i.e. ½ MS, MS and WPM containing various combinations

of IBA (0.1-2.0 mgl^{-1}) and NAA (0.1-2.0 mgl^{-1}) were used. Observations on rooting (%), number of roots/ explant and average length of roots (mm) were recorded after four weeks.

Results and Discussion

The results of present investigation are described under appropriate heads supplemented with tables and plates.

4.1.1 Effect of medium supplemented with growth regulators on necrotic cultures

Perusal of data (Table 1) shows that M_2 induced least necrotic culture (13.16 %), which was statistically different from M_3 and M_1, which induced necrotic cultures of 14.34 per cent and 16.34 per cent respectively. BAP (1.5mgl^{-1}) and IBA (0.01mgl^{-1}) proved best combination by inducing least necrotic culture induction of 5.83 per cent which was statistically best when compared with other combinations in terms of induction of necrotic cultures during establishment stage. A significant interaction between various media and growth regulator combinations was observed for necrotic culture induction during establishment stage of. M_2 medium supplemented with BAP (1.5mgl^{-1}) and IBA (0.01mgl^{-1}) proved best by inducing least necrotic cultures of 3.05 per cent followed by 6.50 per cent in M_3 medium fortified with BAP (1.5mgl^{-1}) and IBA (0.01mgl^{-1}). M_1 medium supplemented with BAP (3.0mgl^{-1}) and IBA (2.0mgl^{-1}) induced maximum necrotic cultures (27.05 %), which was statistically far more than any other treatment combination.

These results are in conformity with earlier observations made by Liete *et al* (1997), De Paoli (1989), who reported Murashige and Skoog medium as the best medium for culture initiation resulting in the highest establishment (%) with least necrotic cultures. Although there is considerable work done on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their *in vitro* effects (Bairu *et al* 2009). But a variation observed in necrosis at various combinations of growth regulators has been credited to medium composition and growing conditions by Bairu *et al* (2009).

Table 1: Effect of media type and growth regulators (mgl^{-1}) on necrotic cultures (%)

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	12.56	8.33	8.62	9.84
BAP(0.5)+IBA(1.0)	18.19	16.15	18.51	17.62
BAP(0.5)+IBA(2.0)	22.29	23.26	18.94	21.50
BAP(1.5)+IBA(0.01)	7.94	3.05	6.50	5.83
BAP(1.5)+IBA(1.0)	16.37	7.10	13.45	12.31
BAP(1.5)+IBA(2.0)	19.84	16.34	18.33	18.17
BAP(3.0)+IBA(0.01)	9.50	10.44	6.71	8.88
BAP(3.0)+IBA(1.0)	19.56	17.02	18.16	18.25
BAP(3.0)+IBA(2.0)	27.05	22.27	24.30	24.54
Control	10.08	7.67	9.86	9.20
Mean	16.34	13.16	14.34	
C.D($p \leq 0.05$)	**Medium (A)=0.453, GR's (B)=1.098, A×B=1.649**			

4.1.2 Effect of medium supplemented with growth regulators on explant establishment

Data in Table 2 reveal that both media and growth regulator had significant effect on establishment (%). Highest establishment of 23.55 per cent was achieved by using M_3 medium, which was significantly higher as compared to M_2 and M_1. The data clearly shows that establishment percentage was highest using BAP ($1.5mgl^{-1}$) and IBA ($0.25mgl^{-1}$) and this combination differed significantly from all other combinations. Interaction studies between medium and growth regulators revealed that the highest explant establishment of 52.80 per cent was achieved from M_2 medium fortified with BAP ($1.5mgl^{-1}$) and IBA ($0.25mgl^{-1}$) {Fig 2}. M_1 medium fortified with BAP ($0.5mgl^{-1}$) and IBA ($0.5mgl^{-1}$) resulted in the lowest establishment (%).Variations in per cent establishment of explants with different doses of auxin and cytokinin during micropropagation are in conformity with the reports given by Fan and Jiang (1993) in apple; Mondal *et al* (1994) in *Carica papaya*; Caboni *et al* (1999) in pear; Chakravarty and Goswami (1999) in *Citrus acida;* Akbar *et al* (2003) in pineapple and Canli and Tian (2008) in sweet cherry. Different media have been tried earlier for establishment of plant species by various workers and reported varied

results in terms of establishment percentage. Peer *et al* (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir Grossa using Murashige and Skoog medium over Driver and Kuniyuli medium, Woody Plant Medium and Knop's macro and MS micro-organics medium independent of growth regulators concentration. *In vitro* effects of growth regulator on overall establishment of explant has been reported to be influenced by growth medium composition, growing conditions and genotype (Bairu *et al* 2009, Karimpour *et al* 2013).

Table 2: Effect of media type and growth regulators (mg/l) on explant establishment (%)

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	21.32	25.05	16.61	20.99
BAP(0.5)+IBA(0.25)	0.40	9.11	25.04	11.52
BAP(0.5)+IBA(0.5)	0.05	0.21	12.47	4.24
BAP(1.0)+IBA(0.01)	11.12	26.68	50.14	29.31
BAP(1.0)+IBA(0.25)	8.36	14.19	41.49	21.35
BAP(1.0)+IBA(0.5)	5.29	11.29	11.72	9.43
BAP(1.5)+IBA(0.01)	18.82	26.36	31.27	25.48
BAP(1.5)+IBA(0.25)	11.06	52.80	27.30	30.39
BAP(1.5)+IBA(0.5)	5.09	18.71	14.30	12.70
Control	5.67	10.40	5.12	7.063
Mean	8.72	19.48	23.55	
C.D($p \leq 0.05$)	**Medium (A)=0.881, GR's (B)= 1.608, A×B=2.786**			

4.1.3 Shoot proliferation

Established explants of Kainth obtained from establishment stage were transferred to shoot proliferation media i.e. M_1, M_2 and M_3 containing different levels BAP. Data regarding shoot proliferation was obtained after two sub culturing as shoot growth was very slow initially irrespective of media used and growth regulator level.

From the perusal of Table 3, it is evident that proliferated culture (%) during proliferation stage is certainly affected by type of media and BAP concentration individually as well as in interactive fashion. With regard to media, irrespective of BAP concentration, maximum proliferated culture (72.13 %) was obtained in M_3,

which is significantly higher than proliferated culture (%) obtained in M_2 and M_1. Maximum proliferated cultures (91.13 %) resulted by using BAP at 3.0mgl^{-1}, which is statistically at par with proliferated culture (%) obtained at BAP (1.5mgl^{-1}). Regarding interaction between type of media and BAP concentration, maximum proliferated cultures (95.30 %) were obtained by using M_3 fortified with BAP (3.0mgl^{-1}) {Fig 3}.

Data in Table 4 clearly shows that with increase in level of BAP, there is increase in number of shoots produced per explant irrespective of media used. Maximum number of shoots per explant was observed in M_3 (4.39) which was followed by M_2 (3.61) and M_1 (2.63). All media differ significantly in terms of production of number of shoots per explant. The highest number of shoots per explant i.e. 4.78 was obtained with BAP (3.0mgl^{-1}) and it was followed by 1.5mgl^{-1} BAP (4.43). Interaction effect revealed that there is significant interaction between media and growth regulator concentration on number of shoots produced per explant in Kainth. M_3 medium fortified with BAP (3.0mgl^{-1}) resulted in maximum number of shoots per explant (6.28) followed by 5.75 in M_3 supplemented with BAP (1.5mgl^{-1}).

Shoots of most desirable length (40.86 mm) were obtained on M_3 medium followed by 38.86 mm on M_1 medium (Table 5). From the perusal of table 5, shoot length clearly decreases with increase in level of BAP and shoots of maximum length were obtained when no growth regulator was added to medium i.e. control. Average shoot length produced at 0.5mgl^{-1} BAP (41.79 mm) and control (42.19) were statistically at par but differ significantly from average shoot length produced at 1.5 mgl^{-1} BAP (37.20 mm) and 3.0mgl^{-1} BAP (33.22 mm). There was no significant interaction between type of media and growth regulator level on average length of shoots although maximum length of shoots was obtained when basal M_3 medium i.e. control was used during shoot proliferation.

These findings are in conformity with those of Dwivedi and Bist (1999) in *P. pyrifolia* cv. Gola and Kadota *et al* (2001) in Japanese pear cultivar Hosui, who reported superiority of WPM over other media with respect to proliferation rate. BAP level was found to effect significantly per cent proliferation, shoots per explant and shoot length and these results are in conformity with studies reported by Dwivedi and Bist (1999), Sedlak and Paprstein (2003), Cosac and Frasin (2008), Karimpour *et al* (2013), Hassanen and Gabr (2012), Ruzic *et al* (2011), Isikalan *et al* (2011) and Soni *et al* (2011). Hu and Wang (1983) reported that cytokinins, especially BAP stimulated axillary bud development but at higher concentration shoot elongation was

suppressed. Similarly, higher number of shoots per explant during proliferation stage on M_2 as compared to M_1 has been reported by Hassan (2012) in Le Conte pear, Tange *et al* (2008) in Bartlett pear and Mustafa *et al* (2013) on fig due to higher nutrient concentration.

Table 3: Effect of media type and growth regulator level (mgl-1) on proliferated cultures (%)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	60.03	71.43	66.65	66.04
BAP(1.5)	84.59	92.51	92.35	89.82
BAP(3.0)	86.44	91.64	95.30	91.13
Control	13.93	25.49	34.23	24.55
Mean	61.25	70.27	72.13	
C.D($p \leq 0.05$)	**Medium (A)= 1.551 , BAP (B)= 1.790 , A×B=3.101**			

Table 4: Effect of media type and growth regulator level (mgl^{-1}) on number of shoots

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	2.76	2.59	2.91	2.75
BAP(1.5)	2.92	4.61	5.75	4.43
BAP(3.0)	3.15	4.92	6.28	4.78
Control	1.68	2.33	2.62	2.21
Mean	2.63	3.61	4.39	
C.D($p \leq 0.05$)	**Medium (A)= 0.141 , BAP (B)= 0.163 , A×B=0.282**			

Table 5: Effect of media type and growth regulator level (mgl^{-1}) on av. length of shoots (mm)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	42.10	40.31	42.97	41.79
BAP(1.5)	39.05	33.53	39.01	37.20
BAP(3.0)	31.02	31.37	37.26	33.22
Control	43.27	39.11	44.20	42.19
Mean	38.86	36.08	40.86	
C.D($p \leq 0.05$)	**Medium (A)= 1.924 , BAP (B)= 2.222 , A×B=NS**			

4.1.4 *In vitro* rooting

The *in vitro* regenerated shoots during shoot proliferation were transferred to various rooting media supplemented with different levels of IBA and NAA. Data from Table 6 reveal that the highest rooting of 5.22 per cent was observed on M_1 medium, which is significantly different from rooting percentage observed on M_2 and M_3. Irrespective of media, IBA ($0.1mgl^{-1}$) induced higher rooting (1.58%) as compared to IBA ($1.0mgl^{-1}$). The interaction effect of treatment combination of M_1 fortified with IBA ($0.1mgl^{-1}$) resulted in best rooting response (13.34 %) {Fig 4}, when compared to M_3 fortified with IBA ($0.1mgl^{-1}$).

Data in Table 7 clearly reveals that the highest number of roots per explant (1.25) was produced in M_1, which is significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (2.01) was obtained by using IBA at $0.1mgl^{-1}$ irrespective of media used. M_1 medium fortified with IBA ($1.0mgl^{-1}$) resulted in significantly higher number of roots per explant (2.79) than what was obtained in M_1 medium fortified with IBA ($0.1mgl^{-1}$).

Perusal of data in Table 8 clearly reveals that there is significant effect of rooting media and IBA levels on average length of roots. Irrespective of IBA levels, M_1 induced average root length of 11.00 mm as compared to 7.79 mm on M_3 and 7.04 mm on M_2 medium. While as IBA at 0.1 mgl^{-1} resulted in maximum average root length of 27.41 mm, which is significantly higher than 7.03 mm observed at IBA ($1.0mgl^{-1}$). Interaction effect revealed that roots of maximum length were produced in

M_3 medium fortified with IBA (0.1mgl^{-1}) having average length of 31.15 mm, which is significantly higher than other treatment combinations.

As compared to IBA, NAA induced higher rooting irrespective of type of rooting media used (Table 9). Data from table 9 clearly reveals that M_1 induced the highest rooting (13.49 %) in Kainth. NAA at 1.0 mgl^{-1} resulted in maximum rooting (24.83 %), which is significantly higher than 22.35 per cent obtained at NAA (0.1mgl^{-1}). A treatment combination of M_1 with NAA (1.0mgl^{-1}) resulted maximum rooting of 29.61 per cent (Fig 5).

With regard to effect on number of roots per explant using different types of rooting media fortified with various levels of NAA showed that M_1 resulted in maximum number of roots per explant. From the perusal of data in table 10, significantly more number of roots per explant (3.07) were observed using NAA (1.0mgl^{-1}), irrespective of media. M_1 fortified with NAA (1.0mgl^{-1}) resulted in the highest number of roots per explant (3.40).

Data in Table 11 shows that average length of roots in Kainth is significantly affected by rooting media and NAA levels. As far as media is concerned, M_3 resulted in longer roots (11.04 mm) irrespective of NAA level. NAA at 0.1 mgl^{-1} resulted in significantly longer roots (20.53 mm) when compared to 16.97 obtained at NAA (1.0mgl^{-1}). A treatment combination of M_3 fortified with NAA (0.1mgl^{-1}) resulted in maximum average length of roots (22.97 mm) which is followed by 21.20 mm in M_3 medium fortified with NAA (1.0mgl^{-1}). These two treatments were statistically at par in terms of controlling average root length.

Thakur (2004) and Thakur and Kanwar (2008) also reported better rooting response of Kainth as compared to scion varieties. Like the multiplication rate, rooting ability being genotype dependent (Sharma *et al* 2007) and rootstocks usually root with greater ability than scions (Dobránszky and Teixeira da Silva, 2010). The mineral concentration in the culture medium affects rooting characteristic and some researchers have proposed that reduction of salt strength to half strength improved rooting (Dimassi-Theriou and Economou, 1993). The reason behind increasing rooting rate on half strength culture medium might be due to a disorder in carbohydrate to nitrogen in nutrient medium, which lead to decreasing nitrogen level in shoot and then improving rooting rate, initiation roots, increasing root number and lengths (Fotopoulos and Sotiropoulos 2005). Higher rooting response in Kainth using NAA than IBA is in conformity with Thakur (2004), Thakur and Kanwar (2008).

Better rooting response of pear genotypes with NAA is in concordance to the findings of Singha (1980) who preferred NAA over IBA for inducing roots in *P. communis* cv. Seckel to avoid the basal callus formation. Reed (1995) also found that some pear genotypes rooted better on NAA than on IBA. Too high an auxin concentration in rooting media is undesirable as it leads reduction in rooting by inducing basal callus formation (Lane 1979) or by inhibiting the root elongation (Thimann 1977). This may be the reason for poor rooting response at higher auxin concentration in the present study.

Table 6: Effect of media type and IBA level (mgl^{-1}) on rooting (%)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	13.34	9.04	9.36	10.58
IBA (1.0)	7.55	0.00	0.00	2.52
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	5.22	2.26	2.34	
C.D($p \leq 0.05$)	**Medium (A)= 0.705, IBA(B)= 0.815, A×B=1.411**			

Table 7: Effect of media type and IBA level (mgl^{-1}) on number of roots per explant

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	2.20	2.01	1.81	2.01
IBA (1.0)	2.79	0.00	0.00	0.93
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	1.25	0.50	0.45	
C.D($p \leq 0.05$)	**Medium (A)= 0.057, IBA(B)= .066, A×B=.114**			

Table 8: Effect of media type and IBA level (mgl^{-1}) on average root length (mm) of micro-shoots

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	22.90	28.17	31.15	27.41
IBA (1.0)	21.10	0.00	0.00	7.03
IBA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	11.00	7.04	7.79	
C.D($p \leq 0.05$)	**Medium (A)= 1.226, IBA(B)= 1.416, A×B=2.452**			

Table 9: Effect of media type and NAA level (mgl^{-1}) on rooting (%)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	24.35	23.41	19.30	22.35
NAA (1.0)	29.61	24.94	19.95	24.83
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	13.49	12.09	9.81	
C.D($p \leq 0.05$)	**Media (A)=1.006, NAA(B)=1.162, A×B=2.012**			

Table 10: Effect of media type and NAA level (mgl^{-1}) on number of roots per micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	3.02	2.78	2.43	2.74
NAA (1.0)	3.40	3.22	2.60	3.07
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	1.61	1.50	1.26	
C.D($p \leq 0.05$)	**Media (A)= 0.057, NAA(B)= 0.065, A×B=0.113**			

Table 11: Effect of media type and NAA level (mgl^{-1}) on average length of roots (mm)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	18.23	20.40	22.97	20.53
NAA (1.0)	14.33	15.37	21.20	16.97
NAA (2.0)	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Mean	8.14	8.94	11.04	
C.D($p \leq 0.05$)	**Medium (A)= 0.919, NAA(B)= 1.061, A×B=1.837**			

Fig 1: Forced cuttings of Kainth

Fig 2: Kainth establishment in MS medium fortified with BAP (1.5 mgl^{-1}) + IBA(0.25 mgl^{-1})

Fig 3: Kainth proliferation in WPM fortified with BAP (3.0 mgl^{-1})

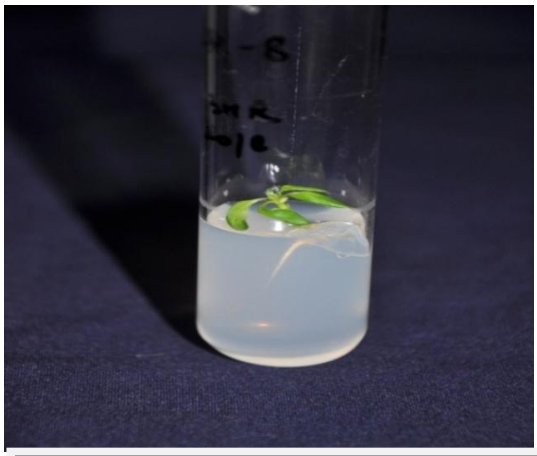

Fig 4: Kainth rooting using ½ MS fortified with IBA (0.1 mgl^{-1})

Fig 5: Kainth rooting using ½ MS fortified with NAA (1.0mgl^{-1})

Plate I: *In vitro* propagation of Kainth

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CHAPTER IV

GENERAL DISCUSSION

Presently pear is next to apple in importance, acreage, production and varietal wealth among temperate fruits in India. It can grow under wider temperature conditions ranging from -26°C when dormant to as high as 47°C during growth period (Chadha 2001). Temperate pear can grow from foothills to high hills (600-2700 m amsl) experiencing 500-1500 chilling hours, while the sub-tropical pears require only 200-300 chilling hours.

Total world pear production reached 20 million metric tons in 2013 (FAO 2013). Pear is grown from warm humid sub-tropical plains to cold dry temperate regions of India occupying an area of 49340 ha with the annual production of 317270 MT (indiastat 2013). The chief pear growing areas are located in Jammu and Kashmir, Himachal Pradesh, Punjab, Uttarakhand, Arunachal Pradesh, Manipur, Mizoram, Nagaland and Tamil Nadu. In Punjab, it ranks 4th among fruit crops in terms of area after citrus, guava and mango and occupies an area of 2787 ha with an annual production of 63040 MT (Anonym 2014). The area can be increased further and cultivation of this crop may prove to be a best alternative for diversification of agriculture.

The necessity to modernize the planting material production technologies of pear has been stimulated by many considerations such as the trends towards increasing the planting densities in field grown trees and the transition to intensive growing systems, which includes the selection of new parents for breeding programmes, the creation and introduction of new cultivars and the modernization of tree habit and pruning. All of these changes have created a demand for more and more quantities of quality planting material. The conventional system of propagating this material is not only time consuming but the material raised is neither uniform nor healthy.

Application of *in vitro* techniques in fruit growing can, therefore, be a viable alternative to circumvent these problems. One such application is shoot tip grafting or micrografting. Micrografting was developed in 1980s (Burger 1984, Jonard, 1986) and consists of the placement of meristem tip or shoots tip explant onto a decapitated rootstock that has been grown aseptically from seed or micropropagated (Hartmann *et al* 2002).

Due to the multiple uses and advantages of micrografting, this technology may be of interest or of potential practical value to technicians, researchers and nursery operators. Professionals in each of these areas would benefit from the introduction of a simpler and more efficient micrografting procedure that is less dependent on mastery of complex techniques and thus will contribute to the practical utility of micrografting as a tool in fruit tree biotechnology.

Keeping in view the popularity and increased demand of Patharnakh by fruit growers there is a need to provide high quality true to type plants of this cultivar on Kainth rootstock for distribution among pear growers. Therefore, the present study was conducted to investigate *in vitro* propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burm f.) Nakai) pear in Tissue culture laboratory in the Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-13. The results obtained during the study are discussed with support of tables and plates as under.

4.1 *In vitro* propagation of Patharnakh and Kainth

4.1.1 Effect of medium on per cent necrotic cultures of Patharnakh.

The data showing effect of different media, i.e. M_1 (half strength Murashige and Skoog medium), M_2 (Murashige and Skoog medium) and M_3 (Woody Plant medium) supplemented with different concentrations of benzylamino purine (BAP) and Indole butyric acid (IBA) on per cent necrotic cultures of Patharnakh (forced and active) are presented in Table 1a and 1b.

Data (Table 1a) reveals that least necrotic culture (4.16 %) was observed on M_2 medium followed by M_1 (8.91 %) and M_3 (9.69 %). All media differ significantly with respect to occurrence of necrosis during establishment stage of Patharnakh (forced) explants (Plate I.A). The lowest necrosis (4.35 %) was observed, when BAP (1.0 mgl^{-1}) along with IBA (0.01 mgl^{-1}) was used. Treatment combination of BAP (1.0 mgl^{-1}) and IBA (0.25 mgl^{-1}) was statistically at par when compared with the best combination i.e. BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Significantly higher per cent necrosis was observed when no growth regulator was used, i.e. under control. Interaction effect between media and growth regulators revealed that the lowest necrosis was observed by using M_2 medium fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by M_2 fortified with BAP (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) and M_3 supplemented with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). These three treatment combinations were at par but differed significantly with rest of treatment combinations for induction of necrosis during establishment stage of *in vitro* propagation of Patharnakh (forced).

Data in Table 1b shows the effect of different media and growth regulator combinations on induction of necrosis in Patharnakh (active) explants. Data clearly revealed that both media and growth regulator combination had significant effect on necrotic culture induction during establishment stage of Patharnakh (active) explants. Least necrosis (10.58 %) was observed on M_2 medium followed by 16.59 per cent on M_3 medium and 20.10 per cent on M_1 medium. All media differ significantly from each other in terms of induction of necrosis. As far as growth regulator combination was concerned, BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved best with least necrosis percentage of 11.76 followed by 12.19 per cent on BAP (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}). These two treatments were at par with respect to each other but differ significantly from rest combinations in terms of induction of necrosis during

establishment stage. Interaction studies depicted that the least necrosis of 2.04 per cent was induced by M_2 supplemented with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by 6.00 per cent on M_2 fortified with BAP (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}). Highest necrosis (27.00 %) was induced by M_1 fortified with BAP (1.5 mgl^{-1}) and IBA (0.5 mgl^{-1}). These results are in conformity with earlier observations made by Liete *et al* (1997) on Bartlett pear, Singha (1980) on Seckel pear, De Paoli (1989) on Conference and Abbe' Fetal pear, who reported Murashige and Skoog medium as the best medium for culture initiation resulting in the highest establishment (%) with least necrotic cultures. Literature on induction of necrotic culture during establishment stage is scanty and inconclusive. Difference in induction of necrotic response in different media tested might be due to difference in composition of basal media. Higher necrosis in Woody Plant Medium may be attributed due to lower total nitrogen content (Mamaghani *et al* 2010). With regard to use of growth regulator, various scientists have reported combinations but variation observed may be due to genotypic difference (Karimpour *et al* 2013). Berardi *et al* (1993) studied the effect of auxins (IBA and NAA) on incidence of shoot tip necrosis during *in vitro* rooting of *P. calleryana*. They found that shoot tip necrosis was not influenced by auxins. Despite considerable research on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their *in vitro* effects (Bairu *et al* 2009). Influence of plant growth regulators on explant necrosis might be influenced by medium composition, growing conditions and genotype (Bairu *et al* 2009). Salisbury and Ross (1992) reported that plant species-specific cytokinin to auxin ratio has role in establishment, proliferation and growth of explant. According to Sha *et al* (1985) apical necrosis in shoots may be related to a mineral nutrients deficiency, e.g., Ca, resulting from the high levels of humidity in the container microenvironment. Minor variation in per cent necrosis as compared to those observed using active explants may be attributed to different physiological state of forced explants and hence different level of sensitivity to 0.1 per cent $HgCl_2$ used for 5 minutes before culture (Bhatt *et al* 2013).

4.1.2 Effect of medium on per cent necrotic cultures of Kainth.

The data showing effect of different media, i.e. M_1, M_2 and M_3 supplemented with different concentrations of benzylamino purine (BAP) and indole butyric acid (IBA) on necrotic culture (%) of Kainth (forced and active) are presented in Table 2a and 2b. Perusal of data (Table 2a) shows that M_2 induced least necrosis (13.16 %), which was statistically different from M_3 and M_1, which induced necrosis to the tune of 14.34 and 16.34 per cent respectively. BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved to be the best combination with least necrosis of 5.83 per cent, which was statistically best when compared with other combinations during establishment stage of Kainth (forced) explants (Plate III.A). A significant interaction between various media and growth regulator combinations was

observed for necrotic culture induction during establishment stage of Kainth (forced) explants. M_2 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved best as it induced least necrosis, i.e. 3.05 per cent followed by 6.50 per cent in M_3 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). M_1 medium supplemented with BAP (3.0 mgl^{-1}) and IBA (2.0 mgl^{-1}) induced maximum necrosis (27.05 %), which was statistically far more than any other treatment combination.

The effect of different media and growth regulator combination on necrotic culture induction in Kainth (active) explants during establishment stage is presented in Table 2b. This data reveal that there exists significant influence of media type and growth regulator concentration on per cent necrosis in Kainth (forced) explants during tissue culture. M_2 medium proved best in terms of least necrotic culture induction of 11.32 per cent followed by M_1 and M_3 which resulted in 14.79 and 14.98 per cent of necrotic cultures respectively. M_1 and M_3 medium were statistically at par in terms of induction of necrosis. The least per cent necrosis (6.35) was observed when BAP (1.5 mgl^{-1}) and IBA (1.0 mgl^{-1}) was used, which was followed by combination of BAP (3.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) resulting in 9.11 per cent of necrotic cultures. Interaction effect between medium and growth regulator revealed that the lowest necrotic cultures (2.10 %) was observed by using M_2 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) followed by M_2 fortified with BAP (3.0 mgl^{-1}) and IBA (0.01 mgl^{-1}). Maximum necrotic culture of 28.66 % was observed in M_1 medium fortified with BAP (0.5 mgl^{-1}) and IBA (2.0 mgl^{-1}). Almost all treatment combinations differ significantly in terms of induction of necrosis during establishment stage of *in vitro* culture of Kainth (active) explants. Variations observed in necrotic culture (%) as affected by different media fortified with different growth regulator combinations in Kainth (forced) and Kainth (active) may be due to their different physiological state and hence different sensitivity level (Bairu *et al* 2009). Although there is considerable work done on the relationship between necrosis and type and concentration of plant growth regulators, there is no definitive explanation for their *in vitro* effects (Bairu *et al* 2009). But a variation observed in necrosis at various combinations of growth regulators has been credited to medium composition and growing conditions by Bairu *et al* (2009). Equilibrium between phenolic compounds and hormones quite affects the success on plant tissue culture (Poessel *et al* 1980). Overall variation in necrosis (%) of forced and active explants of Kainth during establishment stage can also be contributed to different response to mercuric chloride used for sterilization purpose (Bhatt *et al* 2013).

4.1.3 Effect of medium on per cent explant establishment of Patharnakh.

The data pertaining to effect of different media namely half-strength Murashige and Skoog (M_1), full-strength Murashige and Skoog (M_2) and Woody Plant medium (M_3) supplemented with different combinations of benzylamino purine (BAP) and indole butyric

acid (IBA) on explant establishment of Patharnakh (forced and active) are presented in Table 3a and 3b.

Data in Table 3a reveals that both media and growth regulator had significant effect on explant establishment in Patharnakh (forced). Highest explant establishment of 69.68 per cent was obtained using M_2 medium followed by 67.79 per cent on M_1 medium. BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved the best combination of growth regulators and resulted in highest explant establishment of 83.57 per cent and it was significantly higher than all other combinations. The interaction effect showed that highest explant establishment of 96.10 per cent was obtained in M_3 medium supplemented with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) [Plate I.B] and it was at par with M_2 supplemented with BAP (1.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) and M_2 with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}). Lowest explant establishment was obtained on M_3 supplemented with BAP (0.0 mgl^{-1}) and IBA (0.0 mgl^{-1}) i.e. under control and it was significantly lower than all treatment combinations.

Data in Table 3b shows the effect of different media supplemented with various combinations of BAP and IBA on explant establishment using active explants of Patharnakh. Highest explant establishment of 52.65 percent was obtained on M_2 medium followed by M_3 and M_1 medium. As far as growth regulator combination is concerned, BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) proved the best combination for explant establishment followed by BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}). These two treatments were significantly different from rest of the treatment combination. Interaction studies revealed that the highest explant establishment of 96.29 per cent was obtained on M_2`medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}) which was statistically best than rest of treatment combinations (Plate II.A). Lowest explant establishment was obtained on M_1 medium supplemented with BAP (1.0 mgl^{-1}) and IBA (0.25 mgl^{-1}).

Difference in establishment (%) in various media at different combinations of BAP and IBA may be attributed to varying callusing rates and contaminations values as reported by Kozlina and Jelaska (1987). Different media have been tried earlier for establishment of various plant species and varied results have been reported in terms of establishment percentage. Peer *et al* (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir Grossa using Murashige and Skoog medium over Driver and Kuniyuli medium, Woody Plant Medium and Knop's macro and MS micro-organics medium independent of growth regulators concentration. Similar results were obtained by Jones and Hopgood (1979), Snir (1982), Bhojwani *et al* (1984), Shen and Mullins (1984), Erbenova *et al* (2001), Dai-Hong *et al* (2004), Sedlak *et al* (2008), Wang *et al* (1994), Pasqual *et al* (2002a), Hassanen and Gabr (2012), Grigoriadou *et al* (2000), Iglesias *et al* (2004), Thakur and Kanwar (2008a) and Karimpour *et al* (2013). However variations in per cent establishment observed in terms of growth regulator combination from earlier studies might

be due to different genotype, growing conditions, cytokinin to auxin ratio and physiological state of explant (Karimpour *et al* 2013). Rathore *et al* (1991) also reported higher establishment of explants in M_2 medium (MS) supplemented by low level of auxin (IBA) and comparatively higher levels of cytokinin (BAP) in various forest trees. Overall higher establishment (%) of Patharnakh (forced) as compared to Patharnakh (active) can be contributed to higher survival rate as growth chamber-derived explants provided cleaner source material (Bhatt 2008 and Tuia *et al* 2007).

4.1.4 Effect of medium on per cent explant establishment of Kainth.

Data in Table 4a reveal that both media and growth regulator had significant effect on establishment of Kainth (forced) explants. Highest establishment of 23.55 per cent was achieved by using M_3 medium, which was significantly higher as compared to M_2 and M_1. The data clearly shows that establishment percentage was highest using BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) and this combination differed significantly from all other combinations. Interaction studies between medium and growth regulators revealed that the highest explant establishment of 52.80 per cent was achieved in M_2 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) [Plate III.B]. M_1 medium fortified with BAP (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) resulted in the lowest establishment of Kainth (forced) explants.

Explant establishment using Kainth (active) explants as shown in Table 4b was highest when M_2 medium was used during establishment stage of *in vitro* propagation. Explant establishment of 32.14 per cent was achieved in M_2 medium and it was statistically higher as compared to M_1 and M_3 medium. Explant establishment of 52.53 per cent was obtained using BAP (1.5mgl^{-1}) and IBA (0.25mgl^{-1}). This dosage resulted in significantly higher explant establishment as compared to other combinations. The interaction effect showed that the treatment combination of M_2 containing BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) gave maximum per cent explant establishment (63.60) [Plate IV.A] whereas; minimum explant establishment of 10.68 per cent was obtained on M_3 supplemented with BAP (0.0 mgl^{-1}) and IBA (0.0 mgl^{-1}) i.e. control. Variations in per cent establishment of explants with different doses of auxin and cytokinin during micropropagation are in conformity with the reports given by Fan and Jiang (1993) in apple, Mondal *et al* (1994) in *Carica papaya*, Caboni *et al* (1999) in pear, Chakravarty and Goswami (1999) in *Citrus acida* , Akbar *et al* (2003) in pineapple and Canli and Tian (2008) in sweet cherry. Different media have been tried earlier for establishment of plant species by various workers and reported varied results in terms of establishment percentage. Peer *et al* (2013) reported better results in terms of establishment percentage in sweet cherry cv. Bigarreau Noir Grossa using Murashige and Skoog medium over Driver and Kuniyuli medium, Woody Plant Medium and Knop's macro and MS micro-organics medium independent of growth regulators concentration. Similar

results were obtained by Jones and Hopgood (1979), Snir (1982), Erbenova *et al* (2001), Dai-Hong *et al* (2004) and Sedlak *et al* (2008).

4.1.5 *In vitro* shoot proliferation of Patharnakh.

Shoots from established explants were cultured on different media supplemented with varying doses of BAP. Initially, the shoot growth was very slow in all media at all levels of BAP. However, after two subcultures on respective media, two to three axillary shoots arose from the base of cultured shoots.

As evident from Table 5a, per cent proliferated culture in Patharnakh (forced) was significantly affected by type of media and BAP concentrations. The highest proliferated culture per cent (63.19 %) was observed in M_3 followed by 54.78 per cent in M_2, irrespective of BAP concentration. These two media were significantly at par in terms of proliferated cultures. Per cent proliferated culture increased with increase in BAP concentration, non-significantly irrespective of type of medium. There was non-significant interaction between type of media and BAP concentration, although the highest per cent proliferated cultures was obtained in M_3 medium fortified with BAP (2.5 mgl^{-1}) [Plate I.C].

Data in Table 5b show that in all media used, there was considerable increase in number of shoots per explant with increase in BAP concentration upto a level, but further increase in BAP concentration inhibited shoot proliferation significantly in Patharnakh. In Patharnakh (forced), highest number of shoots per explant were obtained on M_3 medium (2.85) followed by M_1 (2.67) and M_2 (2.60). M_1 and M_2 were significantly at par in terms of number of shoots per explant production. Highest number of shoots per explant in Patharnakh (forced) was obtained at BAP (2.5 mgl^{-1}) which was significantly higher than at BAP (0.5 mgl^{-1}) and BAP (5.0 mgl^{-1}). There was significant interaction between various media and growth regulator concentration for number of shoots per explant produced in Patharnakh (forced). Maximum number of shoots per explant (3.80) was produced in M_3 supplemented with BAP (2.5 mgl^{-1}), followed by 3.66 in M_1 supplemented with BAP (2.5 mgl^{-1}).

Shoot length (mm) was also significantly influenced by type of media and growth regulator levels in Patharnakh (forced) during proliferation stage of *in vitro* propagation. From the perusal of Table 5c, it is evident that maximum average length of shoots (49.71 mm) was obtained in M_3 medium, which was significantly higher than 46.72 mm in M_1 and 36.83 mm in M_2 medium. Maximum length of shoots (49.44 mm) were produced at BAP (0.5 mgl^{-1}) followed by 47.67 mm in control. These two treatments were at par but were significantly higher as compared to BAP (0.5 mgl^{-1}) and BAP (5.0 mgl^{-1}). Interaction studies between media and growth regulator revealed that there was significant interaction between them. M_3 medium supplemented with BAP (0.5 mgl^{-1}) resulted in maximum average length of shoots (53.75 mm) followed by M_1 containing no growth regulator and M_1 fortified with BAP (0.5 mgl^{-1}).

Perusal of data in Table 6a clearly revealed that proliferated culture percentage was highest in M_3 medium (56.85), which is significantly higher than 51.28 % in M_2 and 51.11 % in M_1 medium. In Patharnakh (active), proliferated culture percentage increased significantly with increase in concentration of BAP. There was significant interaction between type of media and BAP concentration in determining the proliferated culture (%) in Patharnakh (active) during proliferation stage. M_3 containing BAP (5.0 mgl^{-1}) resulted in significantly highest proliferated cultures (79.47 %) [Plate II.B].

Similarly in Patharnakh (active), number of shoots per explant showed positive relation with BAP concentration upto a level, after which further increase in BAP concentration inhibited shoot proliferation (Table 6b). Maximum number of shoots per explant (2.86) was produced at BAP (2.5 mgl^{-1}) which was significantly higher than 2.60 at BAP (5.0 mgl^{-1}) and 2.50 at BAP (0.5 mgl^{-1}). M_3 medium resulted in maximum number of shoots per explant (2.53) followed by M_2 (2.38) and M_1 (2.14). Interaction effect revealed that there is no significant interaction between media and growth regulator concentration on number of shoots produced per explant in Patharnakh (active). M_2 fortified with BAP (2.5 mgl^{-1}) resulted in maximum number of shoots per explant (3.08) followed by 2.84 in M_3 supplemented with BAP (5.0 mgl^{-1}).

Average length of shoots in Patharnakh (active) also varied with type of media and change in growth regulator level (Table 6c). There was decrease in average length of shoots with an increase in level of BAP. Maximum shoot length (44.43 mm) was produced in M_3 media which was significantly different and higher as compared to shoot length produced in M_1 (40.56 mm) and M_2 (33.53 mm). Shoots of the most desirable length (45.04 mm) was produced in media having no growth regulator i.e. control followed by media supplemented with BAP (0.5 mgl^{-1}). Shoot length was also influenced significantly by considering media and growth regulator level in interactive manner. Maximum average shoot length (51.33 mm) was obtained in M_3 medium containing no growth regulator (basal M_1 medium) i.e. control. It was significantly higher as compared to all treatment combinations.

A number of factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explants development stage, etc. affect shoot proliferation, shoot length and shoots per explant (Bhat *et al* 2012). An initial lag period due to slow growth of cultures has been reported in *Pyrus pyrifolia* (Bhojwani *et al* 1984, Thakur 2004) and *P calleryana* (Stimart and Harbage 1989). Dwivedi and Bist (1999) in *P. pyrifolia* cv. Gola and Thankur and Kanwar (2008a) in *P. pashia* also reported superiority of WPM over MS and ½ MS media. Banno *et al* (1988) also reported better results of shoot proliferation on WPM over ½ MS and ¼ MS media in Japanese pear rootstocks. Thakur (2004) reported that in various species of pear, woody plant medium resulted in the highest shoot proliferation rate; however, it was at par with Murashige and Skoog medium. However,

Ciccotti *et al* (2008) observed better proliferation in MS medium and higher strength MS medium over WPM medium in *Malus* species and this may be due to different genotype (Webster and Jones 19991). Cytokinins overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. The effective concentration of exogenous cytokinin required to reverse apical dominance varies with the culture systems. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by, in decreasing order, kinetin and 2-iso-pentyladenine (2ip) (Hu and Wang 1983). Similarly a positive correlation was reported between BA concentration and shoot multiplication upto a certain BA level by Dwivedi and Bist (1999) in *P. pyrifolia*, Baviera *et al* (1989) in *P. communis*, Thakur and Kanwar (2008a) in *P. pashia*, Shibli *et al* (1997) in *P. syrica*, Karimpour *et al* (2013) in *P pyrifolia* cv. Sebri, Ahmad *et al* (2003) in peach rootstock GF 677 and Silva *et al* (2003) in *Prunus* 'Capdeboscq' rootstocks. Although increasing BAP concentration in shoot multiplication medium enhanced proliferation rate in Patharnakh but shoot length decreased significantly. Increase in shoot proliferation and decrease in shoot length following increase in cytokinin concentration in various media has been reported in *Pyrus* species by Dwivedi and Bist (1999), Berardi *et al* (1993), Stimart and Harbage (1989), Bhojwani et al (1984), Karimpour *et al* (2013) and Thakur and Kanwar (2008a). Stimart and Harbage (1989) and Thakur (2004) reported that shoots of desirable length of *P. pyrifolia* can be obtained by lowering cytokinin level in shoot proliferation medium. The optimal BA concentration for maximal shoot proliferation depends on the cultivar (Lane and McDougald, 1982).

4.1.6 *In vitro* shoot proliferation of Kainth.

Established explants (forced and active) of Kainth obtained from establishment stage were transferred to shoot proliferation media i.e. M_1, M_2 and M_3 containing different levels of growth regulator namely BAP. Data regarding shoot proliferation which comprised of per cent proliferated cultures, number of shoots per explant and average length of shoots was obtained after two sub cultures as shoot growth was very slow initially irrespective of media used and growth regulator level.

From the perusal of Table 7a, it is evident that proliferated culture percentage in Kainth (forced) during proliferation stage is certainly affected by type of media and BAP concentration individually as well as in interactive fashion. With regard to media, irrespective of BAP concentration, maximum proliferated culture (72.13 %) was obtained in M_3, which is significantly higher than proliferated culture percentage obtained in M_2 and M_1. Maximum proliferated cultures (91.13 %) were obtained by using BAP at 3.0 mgl^{-1}, which is statistically at par with proliferated culture (%) obtained at BAP (1.5 mgl^{-1}). Regarding interaction between type of media and BAP concentration, maximum proliferated cultures (95.30 %) were obtained by using M_3 fortified with BAP (3.0 mgl^{-1}) [Plate III.C].

Data in Table 7b clearly shows that with increase in level of BAP, there is increase in number of shoots produced per explant irrespective of media used. Maximum number of shoots per explant in Kainth (forced) was observed in M_3 (4.39) which was followed by M_2 (3.61) and M_1 (2.63). All media differ significantly in terms of production of number of shoots per explant. The highest number of shoots per explant in Kainth (forced) i.e. 4.78 was obtained with BAP (3.0 mgl^{-1}) and it was followed by 1.5 mgl^{-1} BAP (4.43). Interaction effect revealed that there is significant interaction between media and growth regulator concentration on number of shoots produced per explant in Kainth (forced). M_3 medium fortified with BAP (3.0 mgl^{-1}) resulted in maximum number of shoots per explant (6.28) followed by 5.75 in M_3 supplemented with BAP (1.5 mgl^{-1}).

In Kainth (forced), shoots of most desirable length (40.86 mm) were obtained on M_3 medium followed by 38.86 mm on M_1 medium. The perusal of Table 7c clearly shows decrease in shoot length with increase in level of BAP and maximum shoot length was in control. Average shoot length produced at 0.5 mgl^{-1} BAP (41.79 mm) and control (42.19) were statistically at par but differ significantly from average shoot length produced at 1.5 mgl^{-1} BAP (37.20 mm) and 3.0 mgl^{-1} BAP (33.22 mm). There was no significant interaction between type of medium and growth regulator level on average length of shoots produced in Kainth (forced) although maximum length of shoots was obtained when basal M_3 medium, i.e. control was used during shoot proliferation.

Data in Table 8a clearly depicts that in Kainth (active), percentage of proliferated cultures was significantly affected by various treatment combinations. Maximum proliferated cultures (59.09 %) were obtained on M_3 medium. With respect to BAP concentration, maximum proliferated cultures (64.79 %) were obtained by using BAP (3.0 mgl^{-1}), irrespective of media used. Similarly interaction values show that maximum proliferated cultures (83.19 %) were obtained by employing M_3 medium supplemented with BAP (3.0 mgl^{-1}) [Plate IV.B].

From the perusal of data in Table 8b, it is evident that in Kainth (active), number of shoots per explant is influenced by both treatments, i.e. type of medium and growth regulator concentration. The highest number of shoots per explant (4.10) was obtained when M_3 was used as shoot proliferation medium. M_1 and M_2 resulted in significantly lesser number of shoots per explant in case of Kainth (active). Data in Table 19 clearly reveals that maximum number of shoots was obtained by using higher dose of BAP. Increasing BAP level from 0.5 mgl^{-1} to 3.0 mgl^{-1} resulted in increase in number of shoots per explant from 2.95 to 4.65. There existed a significant interaction between type of media and various levels of BAP by effecting number of shoots produced per explant. In Kainth (active), the highest number of shoots per explant (5.93) was obtained in M_3 medium fortified with BAP (3.0 mgl^{-1}) followed by M_3 supplemented with BAP (1.5 mgl^{-1}).

Data regarding shoot length as influenced in by type of media and various doses of BAP in Kainth (active) are presented in Table 8c. It is clearly evident that shoot length decreased with an increase in BAP dosage although number of shoots per explant increased significantly. Shoots of most desirable length were obtained on M_3 medium, which was significantly higher than on M_2 and M_1 medium. Data in Table 20 reveal that BAP (0.5 mgl^{-1}) is best in terms of production of the most desirable shoot length (41.47 mm), which is at par with control (39.97) containing no BAP. There was a significant interaction between type of media and BAP concentration for average length of shoots produced. Longest shoots (46.32 mm) were obtained in M_3 medium that was free of BAP followed by M_3 fortified with BAP (0.5 mgl^{-1}). These two treatments were significantly at par with respect to each other but were significantly better in terms of producing most desirable shoot length when compared with other treatment combinations.

These findings are in conformity with those of Dwivedi and Bist (1999) in *P. pyrifolia* cv. Gola, Dwivedi and Bist (1997) in *P. pashia*, Banno *et al* (1988), Thakur and Kanwar (2008a) in *P. pashia* and Kadota *et al* (2001) in Japanese pear cultivar Hosui, who reported superiority of WPM over other media with respect to shoot proliferation rate. Variation in proliferation rate of Kainth (forced) and Kainth (active) using WPM might be due to different level of maturity of explant (Salisbury and Ross 1992). Benzylaminopurine (BAP) level was found to effect significantly proliferation rate, shoots per explant and shoot length and these results are in conformity with studies reported by Dwivedi and Bist (1997, 1999), Barardi *et al* (1993), Stimart and Harbage (1989), Bhojwani *et al* (1984), Sedlak and Paprstein (2003), Cosac and Frasin (2008), Lane (1979a), Shibli *et al* (1997), Karimpour *et al* (2013), Hassanen and Gabr (2012), Ruzic *et al* (2011), Isikalan *et al* (2011), Ahmad *et al* (2003) and Soni *et al* (2011). Hu and Wang (1983) reported that cytokinins, especially BAP stimulated axillary bud development but at higher concentration shoot elongation was suppressed. The deviation in effect of BAP level on proliferation rate, shoot length and shoots per explant in Kainth (forced) and Kainth (active) might be due to different cytokinin to auxin ratio present in explant (Salisbury and Ross 1992, Karimpour *et al* 2013). Similarly, higher number of shoots per explant during proliferation stage on M_2 as compared to M_1 has been reported by Hassan (2012) in Le Conte pear, Tange *et al* (2008) in Bartlett pear, Baaya (2002) on date palm, Mustafa *et al* (2013) on fig due to higher nutrient concentration.

4.1.7 *In vitro* rooting of Patharnakh

The *in vitro* regenerated shoots (> 30 mm) obtained during shoot multiplication stage of Patharnakh (forced and active) were separated from the shoot clump. These separated shoots were transferred to rooting media, i.e. M_1, M_2 and M_3 containing different growth regulators namely IBA and NAA at different concentrations. Data on the effect of type of media and plant growth regulator on per cent rooting, number of roots per explant and

average length of roots was calculated. NAA did not induce any rooting in Patharnakh (forced and active) irrespective of media used (data not shown). However, very little rooting was obtained by supplementing rooting media with IBA. Thakur (2004) and Thakur and Kanwar (2008a) also failed to induce rooting in Patharnakh using NAA in both solid and liquid media.

Data in Table 9a reveal that type of medium and growth regulator concentration namely IBA had a significant effect on per cent rooting of Patharnakh (forced). In general, better rooting was observed in M_1 having rooting percentage of 3.55 followed by 1.70 % in M_2 and 1.15 % in M_3. All media differed significantly in inducing rooting in Patharnakh (forced). Significantly higher rooting percentage (8.13) was obtained at IBA (1.0 mgl^{-1}) than at IBA (1.5 mgl^{-1}). Rooting in Patharnakh was not obtained at either higher levels of IBA (≥2 mgl^{-1}) or IBA (<1.0 mgl^{-1}). An interaction between type of medium and IBA concentration on rooting (%) was significant for Patharnakh (forced). The highest rooting response (10.16 %) was obtained in M_1 fortified with IBA (1.0 mgl^{-1}) [Plate I.D].

The data in Table 9b shows that in Patharnakh (forced), irrespective of growth regulator concentrations, statistically higher number of roots were obtained in M_1 (0.80) than in M_2 (0.28) and M_3 (0.24). An average of 1.66 roots per explant was obtained at IBA (1.0 mgl^{-1}), which was statistically higher than 0.53 roots per explant obtained at IBA (1.5 mgl^{-1}). An interaction between various media and IBA concentration on roots per explant was significant resulting in 2.38 roots per explant on M_1 medium supplemented with IBA (1.0 mgl^{-1}) in Patharnakh (forced).

Table 9c represents the data on average length of root (mm) as affected by change in rooting media and various levels of IBA (mgl^{-1}) in Patharnakh (forced). The maximum average root length (7.59 mm) was produced in M_1 medium, which was statistically higher as compared to M_3 (5.71 mm) and M_2 (4.80 mm) medium. An average root length of 22.82 mm was produced irrespective of media when IBA (1.0 mgl^{-1}) was used. Interaction effect revealed that roots of maximum length were produced in M_3 medium fortified with IBA (1.0 mgl^{-1}) having average length of 28.57 mm, which was significantly higher than other treatment combinations. No rooting was observed in Patharnakh (forced) irrespective of media at various levels of NAA.

Shoots produced during shoot proliferation stage of Patharnakh (active) were cultured on rooting media supplemented with different levels of IBA and NAA. NAA didn't induce any rooting in Patharnakh (active) irrespective of media used (data not shown). From Table 10a, it is clear that use of particular media and level of IBA certainly effect rooting percentage of Patharnakh (active). Maximum rooting of 3.08 per cent was obtained on M_1 medium followed by 1.04 per cent on M_2 and 0.96 per cent on M_3 medium. IBA at 1.0 mgl^{-1} induced maximum rooting (6.40 %), which was significantly higher than 2.09 per cent obtained at IBA (1.5 mgl^{-1}).

The interaction effect of the treatment combination of M_1 and IBA (1.0 mgl^{-1}) resulted in maximum rooting percentage of 9.16 [Plate II.C].

Perusal of data in Table 10b reveals that there is significant effect of type of rooting media and IBA levels on number of roots produced per explant in Patharnakh (active). The highest number of roots per explant (0.86) was produced in M_1, which was significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (1.94) was obtained by using IBA at 1.0mgl^{-1}. M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant than what was obtained in M_2 medium fortified with IBA (1.0 mgl^{-1}).

Data in Table 1 shows that M_1 rooting medium resulted in longer average root length of 7.04 mm than M_3 (6.63 mm) and M_2 (5.04 mm) medium in Patharnakh (active). The longest average root length was produced by using IBA at 1.0 mgl^{-1}. The interaction effect of M_3 and IBA (1.0 mgl^{-1}) resulted in maximum average root length of 33.13 mm followed by 25.20 mm in M_2 fortified with IBA (1.0 mgl^{-1}).

Patharnakh has been reported more difficult to root by Bhojwani *et al* (1984) and Thakur (2004). Yeo and Reed (1995) investigated several methods and found rooting frequencies of 28 to 100 per cent for 49 genotypes, depending on the treatment and genotype. Out of the 49 genotypes, eight failed to root in all the experiments, this included *P. pyrifolia* and *P. pashia*. Since various media used for rooting were solidified with agar and thus poor rooting response may be due to poor aeration (Torres 1988). Variation in rooting response among the *Pyrus* genotypes may be due to difference in auxin uptake and metabolism (Beraldi *et al* 1993). Similarly, Kohlenbach and Wernicke (1978) and Lane (1979b) reported that agar can have a suppressive effect on rooting response of *Pyrus* species. Huettman and Preece (1993) suggested that rooting of microshoots during micropropagation of various plant species proves difficult because of carry over effect of cytokinin used during proliferation stage. Better results in terms of per cent rooting on ½ MS are in conformity with Marino (1984) in Bartlett pear, Viseur (1987) in Doyenne' du' Comice, Conference, Durondeau and Professeur Molon pear, Baviera *et al* (1989) in Conference pear, Bartish *et al* (1994) in *Pyrus communis* seedling, Thakur and Kanwar (2008a) in various *Pyrus* species, Dwivedi and Bist (1999) in Gola pear, Liaw *et al* (1992) in *P. kawakamii*, Dwivedi and Bist (1997) in *P pashia* and Hassanen and Gabr (2012) in *P. betulaefolia*, Fotopoulos and Sotiropoulos (2005) in PR 204/84 peach rootstock. The promotory effect of a diluted mineral solution on rooting can be explained by the reduction of nitrogen concentration (Al-Bahir *et al* 1999). Kikas *et al* (2006) also reported that high salt concentration of MS promoted active proliferation, but the shoots remained too short to induce rooting during rooting stage, but with low salt concentration of MS, the proliferation rate was lower but shoots elongated enough for better rooting. NAA was found to induce higher rooting percentage at lower concentrations than IBA except in

Patharnakh (forced and active). Singha (1980) reported that NAA avoid the basal callus formation and preferred NAA over IBA for inducing roots in *P. communis* cv Seckel. Zimmerman (1983) and Rai *et al* (2010) reported that for rooting, concentration of mineral salts and sugars should be reduced to half or less of the concentration used for proliferation. Auxin is essential for root initiation and NAA is the most effective auxin for induction of root regeneration. Therefore, NAA is mostly used for inducing rooting followed by IBA, IAA and 2,4-D (Hu and Wang 1983, Mosleh *et al* 2012). Lane (1979a) observed that high concentration of auxin induce callus at the shoot base which inhibits normal root development. Thimann (1977), Werner and Boe (1980) and Thakur and Kanwar (2008b) reported that high auxin is undesirable for root elongation phase and hence inhibited by high concentration of auxin. Hu and Wang (1983) reported that when the auxin concentration is too high, callus would form at the shoot base thus inhibiting normal root development. Variation in rooting response in various media at same auxin level was reported by Novak and Juvova (1983) because the effect of auxins on rooting of shoots depends on the mineral composition of nutrient media. The effect of mineral concentration of the culture medium on rooting can be attributed to the participation of inorganic ions in processes regulating hormonal balance (Amzallag *et al* 1992).

4.1.8 *In vitro* rooting of Kainth.

The *in vitro* regenerated shoots during shoot proliferation of Kainth (forced and active) were transferred to various rooting media supplemented with different levels of IBA and NAA. As compared to Patharnakh, Kainth (forced and active) showed good response to rooting. Data from Table 11a reveal that the highest rooting of 5.22 per cent was observed on M_1 medium, which is significantly different from rooting percentage observed on M_2 and M_3. Irrespective of media, IBA (0.1 mgl^{-1}) induced higher rooting (10.58 %) as compared to IBA (1.0 mgl^{-1}). The interaction effect of treatment combination of M_1 fortified with IBA (0.1 mgl^{-1}) resulted in best rooting response (13.34 %) in Kainth (forced) [Plate III.D], when compared to M_3 fortified with IBA (0.1 mgl^{-1}).

Data in Table 11b represents the effect of rooting media and IBA levels (mgl^{-1}) on number of roots per explant in Kainth (forced). The highest number of roots per explant (1.25) were produced in M_1, which is significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (2.01) was obtained by using IBA at 0.1mgl^{-1}, irrespective of media used. M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant (2.79) than what was obtained in M_1 medium fortified with IBA (0.1 mgl^{-1}).

Perusal of data in Table 11c clearly reveals that there is significant effect of rooting media on average length of roots in Kainth (forced). Irrespective of IBA levels, M_1 induced average root length of 11.00 mm as compared to 7.79 mm on M_3 and 7.04 mm on M_2

medium. While IBA at 0.1 mgl^{-1} resulted in maximum average root length of 27.41 mm, which is significantly higher than 7.03 mm observed at IBA (1.0 mgl^{-1}). Interaction effect revealed that roots of maximum length were produced in M_3 medium fortified with IBA (0.1 mgl^{-1}) having average length of 31.15 mm, which is significantly higher than other treatment combinations.

As compared to IBA, NAA induced higher rooting in Kainth (forced) irrespective of type of rooting media used (Table 12a). Data from Table 12a clearly reveals that M_1 induced the highest rooting (13.49 %) in Kainth (forced). NAA at 1.0 mgl^{-1} resulted in maximum rooting (24.83 %), which is significantly higher than 22.35 per cent obtained at NAA (0.1 mgl^{-1}). A treatment combination of M_1 with NAA (1.0 mgl^{-1}) resulted in maximum rooting of 29.61 per cent in Kainth [Plate III.E].

With regard to the effect on number of roots per explant in Kainth (forced) using different types of rooting media fortified with various levels of NAA showed that M_1 resulted in maximum number of roots per explant (Table 12b). The perusal of data in Table 12b showing significantly more number of roots per explant (3.07) were observed using NAA (1.0 mgl^{-1}), irrespective of media. M_1 fortified with NAA (1.0 mgl^{-1}) resulted in the highest number of roots per explant (3.40) in Kainth (forced).

Data in Table 12c shows that average length of roots in Kainth (forced) is significantly affected by rooting media and NAA levels. As far as media is concerned, M_3 resulted in longer roots (11.04 mm) irrespective of NAA level. NAA at 0.1 mgl^{-1} resulted in significantly longer roots (20.53 mm) when compared to 16.97 mm obtained at NAA (1.0 mgl^{-1}). In Kainth (forced), a treatment combination of M_3 fortified with NAA (0.1 mgl^{-1}) resulted in maximum average length of roots (22.97 mm), followed by 21.20 mm in M_3 medium fortified with NAA (1.0 mgl^{-1}). These two treatments were statistically at par in terms of controlling average root length in Kainth (forced).

Thakur (2004) and Thakur and Kanwar (2008a) also reported better rooting response of Kainth over Patharnakh irrespective of growth regulator and media used. Like the multiplication rate, rooting ability being genotype dependent (Sharma *et al* 2007) and rootstocks usually root with greater ability than scions (Dobranszky and Teixeira da Silva 2010). The mineral concentration in the culture medium affects rooting characteristic and some researchers have proposed that reduction of salt strength to half strength improved rooting (Dimassi-Theriou and Economou, 1993). The reason behind increasing rooting on half strength culture medium is might be due to back to a disorder in carbohydrate to nitrogen in nutrient medium, which lead to decreased nitrogen level in shoot and thus improving rooting rate, initiation of roots, increasing root number and length (Fotopoulos and Sotiropoulos 2005). Higher rooting response in Kainth using NAA than IBA is in conformity with Thakur (2004) and Thakur and Kanwar (2008a). Better rooting response of pear

genotypes with NAA is in concordance to the findings of Singha (1980) who preferred NAA over IBA for inducing roots in P. communis cv. Seckel to avoid the basal callus formation. Reed (1995) also found that some pear genotypes rooted better on NAA than on IBA. Mosleh *et al* (2012) also reported better rooting responses in pear and apple using NAA over IBA and IAA. Too high an auxin concentration in rooting media is undesirable as it leads to reduction in rooting by inducing basal callus formation (Lane 1979a) or by inhibiting the root elongation (Thimann 1977). This may be the reason for poor rooting response in Kainth (forced and active) at higher auxin concentration in the present studies.

Data pertaining to the per cent rooting, roots per explant and average length of roots (mm) as affected by type of media and IBA levels in Kainth (active) is presented in Tables 13a, 13b and 13c. The *in vitro* shoots obtained during proliferation stage of Kainth (active) were transferred to various rooting media supplemented with different levels of IBA (mgl^{-1}). Data in Table 13a clearly reveal that rooting percentage was effected significantly by the rooting media. The highest level of rooting (5.67 %) was noticed in M_1 medium, followed by 2.23 per cent in M_2 and lowest (2.10 %) in M_3 medium. Rooting percentage of 10.46 was obtained using IBA (0.1 mgl^{-1}), which was significantly higher than rooting at higher levels of IBA i.e. 2.89 per cent at IBA (1.0 mgl^{-1}). The highest rooting (14.08 %) was obtained in M_1 medium fortified with IBA (0.1 mgl^{-1}) followed by 8.90 per cent in M_2 supplemented with IBA (0.1 mgl^{-1}) [Plate IV.C].

Data in Table 13b represents the effect of rooting media and IBA levels (mgl^{-1}) on number of roots per explant in Kainth (active). The highest number of roots per explant (1.15) was produced in M_1, which is significantly higher than those obtained on M_2 and M_3. Maximum number of roots per explant (1.83) was obtained by using IBA at 0.1 mgl^{-1} irrespective of media used. M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant (2.60) than rest of treatment combinations.

In Kainth (active) roots of maximum length (10.73 mm) were obtained in M_1 medium which is significantly higher than root length achieved in M_3 and M_2 medium (Table 13c). Irrespective of media used, IBA (0.1 mgl^{-1}) resulted in longer roots (27.80 mm) than root length obtained at IBA (1.0 mgl-1). Interaction effect revealed that roots of maximum length (31.10 mm) were produced in M_3 medium fortified with IBA (0.1 mgl^{-1}), which is significantly higher than other treatment combinations.

Tables 14a, 14b and 14c shows the data regarding the effect of type of rooting media and NAA levels on per cent rooting , number of roots per explant and average length of roots (mm) respectively, in Kainth (active). Data in Table 14a clearly reveal that the per cent rooting induced by NAA was higher than rooting induced by IBA and rooting was effected significantly by type of media and level of NAA. The highest level of rooting (12.65 %) was recorded in M_1 medium followed by 11.83 per cent in M_2 and lowest (9.57 %) in M_3 medium.

Rooting percentage of 22.90 was obtained using NAA (1.0 mgl^{-1}) irrespective of type of rooting media, which is significantly at par to per cent rooting obtained at NAA (0.1 mgl^{-1}) i.e. 22.51 %. The highest rooting (27.46 %) was observed in M_1 medium fortified with NAA (1.0 mgl^{-1}) [Plate IV.D] followed by 24.58 per cent in M_2 medium supplemented with NAA (1.0 mgl^{-1}).

In Kainth (active), number of roots per explant was significantly higher by using M_2 medium irrespective of NAA fortification (Table 14b). NAA at 1.0 mgl^{-1} induced maximum number of roots per explant (2.84) followed by 2.63 using NAA (0.1 mgl^{-1}). An interaction between various media and NAA concentration on roots per explant was significant resulting in 3.60 roots per explant on M_2 medium supplemented with NAA (1.0 mgl^{-1}) followed by 3.26 roots per explant in M_1 fortified with NAA (1.0 mgl^{-1}).

Similarly, root length in Kainth (active) was seen to be significantly affected by type of media and NAA level (Table 14c). Statistically longer roots (8.32 mm) were observed on M_1 medium followed by M_2 and M_3, irrespective of NAA dosage. NAA at 0.1 mgl^{-1} resulted in longer average root length of 19.95 mm. Interaction values of average root length (mm) indicate that type of media and NAA levels has significant effect on this parameter in Kainth (active). M_3 medium supplemented with NAA (1.0 mgl^{-1}) resulted in longer roots (23.22 mm) followed by 21.56 mm in M_3 fortified with NAA (0.1 mgl^{-1}).

4.2 Shoot tip grafting of Patharnakh on Kainth rootstock

4.2.1 Standardization of scion length, grafting technique and medium composition for shoot-tip grafting of Patharnakh (*in vitro* shoot tips) on Kainth.

Scions from *in vitro* propagated Patharnakh were grafted using three different length (L_1, L_2, L_3) by two grafting techniques i.e. wedge (G_1) and horizontal (G_2) grafting (Plate V.A and V.B) and allowed to grow in four types of media i.e. MS medium + 20 g/l sucrose (M_1), liquid MS medium + 20g/l sucrose (M_2), MS medium + 40 g/l sucrose (M_3) and liquid MS medium + 40 g/l sucrose (M_4). Observation on aseptic culture (%), grafting success (%) were taken 4 weeks after grafting while as observations regarding necrosis (%), vitrification (%) and vigour (1-3 point scale) were taken 6 weeks after grafting.

The perusal of data from Table 15a shows that scion length has significant effect on per cent aseptic culture. Scion length (5-10 mm) resulted in the highest aseptic culture percentage which is statistically higher than L_1 (<5mm) and L_3 (10-15mm). Regarding grafting technique, horizontal grafting (G_2) resulted in statistically higher aseptic culture percentage as compared to wedge grafting (G_1). With regard to media, M_1 resulted in the highest aseptic culture (%) followed by M_2, M_3 and M_4. All media have significant effect on per cent aseptic culture after shoot tip grafting.

Combined effect of scion length, grafting technique and media as shown in Table 15a contributed significantly towards per cent aseptic culture. Higher per cent aseptic culture

resulted by using L_2 and G_2 (horizontal grafting of 5-10 mm scions) followed by L_1 and G_2 (horizontal grafting of less than 5 mm scions). Regarding scion length × media, highest per cent aseptic culture resulted by employing L_2 and M_1 (MS medium + 20 g/l sucrose containing micrografts using 5-10 mm scions), which is significantly different as compared to all other treatment combinations. With regard to grafting technique × media, statistically higher per cent aseptic culture resulted by using G_2 and M_1 (MS medium + 20 g/l sucrose containing micrografts using horizontal grafting). Interactive effect of scion length, grafting technique and media indicated that the highest per cent aseptic culture resulted by using L_2, G_2 and M_1 medium (84.51%) followed by L_2, G_2 and M_2 (81.37%).

Bhatt (2008) while working with micrografting of apple cv Ambri on M-9 rootstock reported least per cent contamination by using 10 mm scions as compared to 5 and 15 mm long scions. He also advocated use of *in vitro* scions for micrografting because of less contamination as compared to *in vivo* source. Similarly, use of *in vitro* scions of 5 mm length resulted in least contamination (10.21 %). In terms of interaction, least contamination of 9.48 per cent resulted by using *in vitro* scions of 10 mm length grafted on M-9 rootstock kept in root initiation medium, followed by one week in root elongation medium. Grafting method was also observed to influence per cent contamination with least contamination using horizontal grafting followed by wedge on stump grafting (Bhatt 2008). Influence of medium used during micrografting on per cent aseptic culture is supported by findings by Bhatt *et al* (2013) and Bhatt (2008), who also observed highest aseptic culture (%) using Murashige and Skoog semi solid medium with sucrose 3 %. Combined influence of grafting technique and medium composition as observed during current study matches with results obtained by Bhatt (2008), who also reported less contamination by using horizontal grafting and MS medium with sucrose 3 %.

From the data in Table 15b, it is clearly evident that individual effect of scion length is significant on per cent graft success. Maximum graft success (%) was recorded by using L_2 (5-10 mm) scions (Plate VI.A) followed by L_1 (< 5 mm) and L_3 (10-15 mm). Similarly, grafting technique also affected per cent grafting success. G_1 (wedge grafting) resulted in significantly higher graft success as compared to G_2 (horizontal grafting). While finding the effect of media irrespective of scion length and grafting technique, M_2 (MS liquid medium + 20 g/l sucrose) proved statistically the best medium in terms of graft success followed by M_3 (MS medium + 40 g/l sucrose), M_4 (MS liquid medium + 40 g/l sucrose) and M_1 (MS medium + 20 g/l sucrose). M_3, M_4 and M_1 were statistically at par in terms of per cent graft success. The low success while using small scion (<5mm) during shoot-tip grafting may be due to the great friability of meristematic tissues and the difficult to hold them in close contact with cambial surface of the rootstock. The fast growth of the wounded tissues also seems sometimes to strangle the explant under the rapid proliferation of the surrounding cells

and hence slow growth of scion was observed and no elongation was obtained. When large scions were used, it leads to more callus growth at the base of the scion which contributed to graft failure and larger scions also contain more phenolic compounds and hormonal concentrations results in higher polyphenol oxidases and peroxidases activity and hence higher browning and drying of fresh tissue just before and beyond grafting member's integration (Jonard 1986). Thimmappaiah *et al* (2002) observed that graft success was high (79.5 %) when the scion length was greater than 5 mm and it was less (0.05 %) when size of scion was small (3-5 mm). Onay *et al* (2004) reported that graft success increased in micrografted *Pistachio* to 79.25 per cent with increase in size of the microscion upto 6 mm which then decreased significantly to 17.25 per cent, when scion size was increased to more than 10 mm. Similarly Amiri (2006) obtained highest percentage of successful grafts (65.5 %) in cherry with apical bud scion length greater than 6 mm. The relationship between grafting success and scion size has also been reported in other woody species by Navarro *et al* (1975) in citrus, Ghorbel *et al* (1998) in almond, Bhatt *et al* (2013) in apple and Hoa *et al* (2004) in citrus and Saurez *et al* (2005), Singh *et al* (2008) in citrus and Khalafalla and Daffalla (2008) in Gum Arabic. Increased success observed in the larger size scion i.e 5-10 mm may be due to better cambial contact of the scion with rootstock. Bhatt *et al* (2013) reported vertical slit micrografting technique to be superior as compared to horizontal grafting due to complete vascular connection between shoot tips and rootstock in apple. Onay *et al* (2004) also reported better union formation using slit micrografting while as misalignment and graft dislocation was observed in other methods which led to graft failure and high browning/necrotic cultures. Similar results were reported by Wu *et al* (2007) in *Protea cynaroides*, Naz *et al* (2007) in citrus and Hsina and El Mtili (2009) in *Ceratonia siliqua*. Rafail and Mosleh (2010) made comparison between T-budding and cleft grafting (apex shoot-tip) methods on successful grafts of apple and pear and reported that graft success is significantly affected by grafting method employed. Bhatt *et al* (2013) while investigating effect of different media formulations on graft success reported highest micrografting success rate using MS semi-solid media followed by MS liquid media and MS liquid plus vermiculite media. Higher graft success (24.55 and 21.89 %) was obtained, when micrografts were cultured on MS semi-solid media at 3 and 6 per cent sucrose, respectively. Navarro *et al* (1975), Naz *et al* (2007), Kobayashi *et al* 2003, Ioannou *et al* (1991), Rafail and Mosleh (2010) and Singh *et al* (2008) also reported that increasing concentration of sucrose increased percent survival of micrografts. However, Thimmappaiah *et al* (2002) showed no beneficial effect of increasing sucrose concentration on graft success.

Combined effect of scion length × grafting technique on graft success (%) differ significantly with maximum graft success (%) achieved by using L_2 and G_1 followed by L_1 and G_1. Regarding scion length × media, highest graft success (%) resulted by employing L_2

and M_2 although various treatment combinations were statistically at par in terms of graft success. Interactive effect of grafting technique × media revealed that G_1 and M_2 resulted in maximum graft success followed by G_1 and M_4. While as interactive effect of scion length, grafting technique and medium composition on graft success (%) revealed that the highest graft success (33.71 %) was achieved by using L_2, G_1 and M_2. This treatment combination is statistically higher as compared to all other treatment combinations in terms of graft success. Literature on graft success as affected by interaction of scion length, grafting technique and media formulation is scanty and inconclusive. However, Bhatt (2008) reported an interactive effect of grafting methods and various media formulations on graft success in apple. He reported graft success of 29.96 per cent using wedge grafting and Murashige and Skoog liquid medium with sucrose 3 per cent. Similarly highest graft success of 27.88 per cent was achieved in Ambri using *in vitro* scion of 10 mm. Better results in terms of graft success were obtained when rootstock was kept for one week in root initiation medium followed by one week in root elongation medium as compared to one week in root initiation medium followed by two weeks in root elongation medium and one week in root initiation medium (Bhatt 2008). Variations observed in graft success in present study may be due to different genotype and conditions.

Data in Table 15c clearly reveals that scion length, grafting technique and medium composition do have an effect on occurrence of necrosis (Plate VI.B) in shoot tip grafted Patharnakh. Statistically least per cent necrosis resulted by using L_1 (< 5 mm scions) as compared to L_2 (5-10 mm) and L_3 (10-15 mm). Per cent necrosis was observed to be less in G_1 (wedge grafting) grafted plantlets as compared to G_2 (horizontal grafting) grafted plantlets. While finding individual effect of media irrespective of scion length and grafting technique, M_4 (MS liquid medium + 40 g/l sucrose) induced least per cent necrosis while M_1 (MS medium + 20 g/l sucrose) resulted in the highest per cent necrosis. Different media differed significantly with respect to induction of necrosis in grafted plantlets.

The results of combined effect of scion length × grafting technique on per cent necrosis are significant (Table 15c). Least necrosis resulted by employing L_1 and G_2 i.e. horizontal grafting using less than 5 mm scions followed by L_2 and G_1 (wedge grafting using 5-10 mm scions). Whereas combined effect of scion length × media revealed that L_2 and M_4 resulted in per cent least necrosis but this treatment combination is statistically at par with L_1 and M_2. However, combined effect of grafting technique × medium composition does have significant effect on per cent necrosis. Minimum per cent necrosis was observed by employing G_1 and M_4. While as interactive effect of scion length, grafting technique and medium composition revealed that the lowest per cent necrosis was achieved by using L_3, G_1 and M_4 (10-15mm scions grafted by wedge grafting grown in MS liquid medium containing 40g/l sucrose); L_1, G_2 and M_2 (< 5 mm scions grafted using horizontal grafting grown in MS

liquid medium containing 20 g/l sucrose); L_1, G_2 and M_4 (< 5 mm scions grafted using horizontal grafting grown in MS liquid medium containing 40 g/l sucrose); L_2, G_2 and M_4 (5-10 mm scions grafted using horizontal grafting grown in MS liquid medium containing 40 g/l sucrose). These treatment combinations are statistically at par in terms of inducing least necrosis in grafted plantlets. Bhatt (2008) also reported that in apple micrografting, best source of shoot tips was *in vitro* derived shoots and the poorest source was *in vivo* forced shoot tips. Higher browning/necrotic cultures (57.15 %) and browning/ necrosis intensity (3.38) was observed when scions derived from *in vivo* forced tips were used for grafting which decreased to 39.06 per cent and 2.20 per cent, respectively when *in vitro* derived scion tips were used. Deogratias *et al* (1991) reported that the source of shoot tips had an important influence on grafting success and graft development in apricot. The best graft success (69 %) and graft development (58 %) was obtained with shoot tips from *in vitro* growing shoots which decreased significantly to (26 %) and (14.5 %) respectively, when forced budwood were used as source of shoot tips. Similar results were obtained by Baydar and Celik (1999) in grapes, Ghorbel *et al* (1998) in almond and Onay *et al* (2004) in Pistachio. Bhatt *et al* (2013) reported the lowest necrosis/browning (29.33 %) of apple micrografts using vertical slit grafting as compared to 40.36 % using horizontal grafting. Bhatt (2008) reported highest browning/necrotic cultures (40.58 %) during micrografting in apple using MS liquid media plus vermiculite with 6 per cent sucrose, which decreased to (29.83 %) when plants were cultured in MS semi-solid media at 3 per cent sucrose. Bhatt (2008) also reported lowest browning/necrosis per cent of apple micrografts using slit grafting grown in Murashige and Skoog semi-solid medium with sucrose 3 %, while as highest necrosis per cent was observed using horizontal grafting grown in Murashige and Skoog liquid medium plus vermiculite with sucrose 6 per cent. Bhatt (2008) while carrying effect of scion origin, scion length and root developmental stage on browning/ necrosis of apple cv Lal Ambri on M-9 rootstock reported that least browning/necrosis per cent and intensity resulted by using *in vitro* scion of 10 mm as compared to forced shoot tips for grafting on M-9 rootstock kept in root initiation medium for one week followed by one week in root elongation medium. He credited this variation to difference of polyphenol exudation (Deogratias *et al* 1991) which also leads to difference of grafting success achieved using these two scion sources.

The perusal of data in Table 15d revealed the highest per cent vitrification resulted by using L_3 (10-15 mm) scions (Plate VI.C) whereas minimum vitrified plantlets were developed by using L_2 (5-10 mm) scions. L_3 was significantly at par with L_1 in terms of development of vitrified plantlets. Regarding grafting technique, G_1 (wedge grafting) proved significantly better than G_2 (horizontal grafting) by causing less vitrification irrespective of scion length and growing media. Similarly with regard to various media employed, M_3 (MS medium containing 40 g/l sucrose) resulted in less vitrification followed by M_1 (MS medium + 20 g/l

sucrose), M_4 (MS liquid medium + 40 g/l sucrose) and M_2 (MS liquid medium + 20 g/l sucrose).

Combined influence of scion length × grafting technique as shown in Table 15d on per cent vitrification (%) is significant. Minimum vitrification (%) was observed by using L_2 and G_1, which is significantly far better than other treatment combination of scion lengths and grafting techniques. Similarly, combined effect of scion length × media on occurrence of vitrification in grafted plantlets is also significant. In this case, L_2 and M_2 proved the best combination although this treatment combination is statistically at par with L_3 and M_3. With regard to combined effect of grafting technique and media, G_1 and M_3 proved the best in terms of causing least vitrification (%) in grafted plantlets.

Literature on influence of grafting method, scion length, scion origin and medium along with their interaction on vitrification is lacking. However, Bhatt (2008) reported that vitrification is influenced by grafting method and medium composition and their interaction, with least vitrification by using vertical slit grafting as compared to horizontal grafting and wedge on stump grafting in apple. He also observed least vitrification by growing micrografts in MS semi solid medium with sucrose 6 %. Slit grafted plantlets in MS semi solid medium with sucrose 6 % were seen to be least affected by vitrification. Deberg *et al* (1983) and Harada and Murai (1996) reported that vitrification was related to nutrient medium composition. It was especially common when the plant has too much water available. The vitrification rate was always higher in liquid than in solid media. The frequency of vitrification increased on medium containing a low sucrose concentration, as sucrose decreases the water potential. Dziedzic (2004) also obtained better results of cherry micrografts on solid than on liquid medium because of graft-units vitrification and contamination of liquid medium. Similar results on vitrification were observed by Al-Maarri and Al-Ghamdi (1996), Amiri (2006), Bhatt *et al* (2013) and Ghorbel *et al* (1998) in different fruit crops.

Vigour was significantly affected by scion length, grafting technique and media composition individually as well as by their interaction (Table 15e). Scion length influenced vigour significantly with L_2 (5-10 mm scions) resulting in vigorous plantlets. L_2 was statistically at par with L_1 (< 5 mm scions) in terms of vigour of grafted plantlets. G_1 (wedge grafting) resulted in statistically highly vigorous plantlets as compared to G_2 (horizontal grafting) irrespective of scion length and growing media. With regard to media, M_4 (MS liquid medium + 40 g/l sucrose) resulted vigorous plantlets as compared to M_2 (MS liquid medium containing 20 g/l sucrose), M_3 (MS medium containing 40 g/l sucrose) and M_1 (MS medium containing 20 g/l sucrose). All media differed significantly in determining vigour of plantlets, irrespective of grafting technique and scion length. From the perusal of Table 43, it is clearly visible that scion length × grafting technique has significant effect on vigour of

grafted plantlets. Vigour was high in plantlets produced by employing L_2 and G_1 as compared to other treatment combinations. Regarding scion length × media, L_2 and M_4 resulted in highly vigorous plantlets. This treatment was found to be statistically at par with L_1 and M_2 and L_1 and M_4 in terms of vigour of grafted plantlets. With regard to combined effect of grafting technique × media, G_1 and M_4 produced plantlets of high vigour as compared to other treatment combinations. The interactive effect of scion length, grafting technique and media composition on vigour was statistically significant as evident from Table 15e. Highly vigorous plantlets were produced by using L_2, G_1 and M_2 (Plate VI.D) followed by those plantlets employing L_2, G_1 and M_3.

High vigour using *in vitro* scion of 10 mm in apple was also reported by Bhatt (2008) and Bhatt *et al* (2013). Bhatt (2008) reported that vigour of micrografted plantlet is significantly affected by combined effect of scion origin, scion length, grafting technique and rootstock developmental stage. Bhatt *et al* (2013) reported the highest vigorous plantlets resulted by carrying vertical slit grafting technique as compared to wedge and horizontal grafting. Similarly, Murashige and Skoog medium with 3 per cent sucrose resulted in the most vigorous micrografted apple plantlets when compared with other medium formulations (Bhatt 2008). He also reported that most vigorous plantlets resulted when slit grafted and growth in Murashige and Skoog liquid medium with sucrose 3 %. Variations observed in present study in vigour as compared the previous studies may be attributed to different genotype and prevailing conditions.

4.2.2 Standardization of scion length, grafting technique and medium composition for shoot-tip grafting of Patharnakh (forced shoot tips) on Kainth

Scions from forced Patharnakh cuttings maintained under growth chamber were grafted using three different length (L_1, L_2, L_3) by two grafting techniques i.e. wedge (G_1) and horizontal (G_2) grafting and allowed to grow in four types of media i.e. MS medium + 20mg/l sucrose (M_1), liquid MS medium + 20g/l sucrose (M_2), MS medium + 40mg/l sucrose (M_3) and liquid MS medium + 40mg/l sucrose (M_4). Observation on aseptic culture (%), grafting success (%) were taken 4 weeks after grafting while as observations regarding necrosis (%), vitrification (%) and vigour (1-3 scale) were taken 6 weeks after grafting.

As evident from Table 16a, scion lengths, grafting techniques and media composition affect per cent aseptic culture both individually as well as interactive manner during shoot tip grafting of Patharnakh using forced shoot tips. Maximum aseptic culture percentage was achieved by using L_2 (5-10 mm) scions, irrespective of grafting technique and media composition. As far as individual effect of grafting technique is concerned, G_2 (horizontal grafting) resulted in statistically more aseptic cultures as compared to G_1 (wedge grafting). Aseptic culture (%) was also significantly affected by media composition, with M_1 (MS

medium containing 20 g/l sucrose) resulted in maximum aseptic cultures as compared to other media.

Combined effect of scion length × grafting technique on per cent aseptic culture differ significantly with maximum aseptic culture percentage achieved by using L_2 and G_1 followed by L_1 and G_2. Regarding scion length × media, highest aseptic culture (%) resulted by employing L_2 and M_1 followed by L_2 and M_2. Interactive effect of grafting technique × media revealed that G_2 and M_1 resulted in maximum aseptic culture (%) although other treatment combinations were statistically at par with this treatment. While as interactive effect of scion length, grafting technique and medium composition on aseptic culture revealed that the highest aseptic culture (67.21 %) was achieved by using L_2, G_1 and M_1 (Table 16a). This treatment combination is statistically higher as compared to all other treatment combination in terms of aseptic culture (%).

The perusal of data in Table 16b revealed graft success is significantly affected by scion length, grafting technique and media composition. The highest per cent graft success resulted by using L_1 (< 5 mm) under forced scion as compared to L_2 (5-10 mm) under *in vitro* scion source. With regard to grafting technique, G_1 (wedge grafting) resulted in better graft success as compared to G_2 (horizontal grafting) irrespective of scion length and growing media. With respect to growing medium used after carrying shoot-tip grafting, M_2 (MS liquid medium containing 20 g/l sucrose) resulted in significantly higher graft success followed by M_3 (MS medium containing 40 g/l sucrose), M_1 (MS medium containing 20 g/l sucrose) and M_4 (MS liquid medium containing 40 g/l sucrose).

Combined influence of scion length × grafting technique as shown in Table 16b on graft success is significant. Maximum per cent graft success was observed by using L_1 and G_1 (Plate VII.A), which is significantly far better than other treatment combination of scion lengths and grafting techniques. Combined effect of scion length × media on graft success is non-significant, although highest graft success was achieved by using L_2 and M_1. With regard to combined effect of grafting technique and media, G_1 and M_2 proved the best in terms of per cent graft success but various treatment combinations of grafting techniques and media were statistically at par. While as interactive effect of scion length, grafting technique and medium composition revealed that the highest graft success (18.55 %) was achieved by using L_2, G_1 and M_2. This treatment combination is statistically higher as compared to all other treatment combination in terms of per cent graft success.

Graft success (%) using forced shoot tips was less as compared to *in vitro* shoot tips which is in concordance to Bhatt *et al* (2013), Aazami and Hassanpouraghdam (2010), Rafail and Mosleh (2010), Luna *et al* (2002), Deogratias *et al* (1991) and Edriss and Burger (1984). Despite that, *in vivo* derived shoot-tips are larger in size than *in vitro* derived ones, making handling of them easier (Murashige and Skoog 1962). However, they contain more phenolic

compounds and hormonal concentrations results in higher polyphenol oxidases and peroxidases activity and hence higher browning and drying of fresh tissue just before and beyond grafting member's integration (Jonard 1989).

As evident from Table 16c, per cent necrosis was significantly affected by scion length, grafting technique and media composition both individually and in interactive manner. Least per cent necrosis resulted by using L_2 (5-10 mm scions) but necrosis induced by using L_1 (<5 mm) was found to be statistically at par with L_2. Horizontal grafting (G_2) resulted in significantly lower necrosis (%) as compared to wedge grafting (G_1). With regard to media, M_2 (MS liquid medium containing 20 g/l sucrose) proved to be the best and resulted in least necrosis of grafted plantlets. Various media tested differed significantly in terms of necrosis of grafted plantlets (Plate VII.B).

Scion length and grafting technique significantly affected per cent necrosis in interactive manner as evident from Table 16c. Minimum per cent necrosis resulted by using L_1 and G_2 although necrosis induced by treatment combination of L_2 and G_1 was significantly par with L_1 and G_2. Regarding combined effect of scion length and medium composition, least per cent necrosis in grafted plantlets resulted by using L_3 and M_2. Combined effect of grafting technique and medium composition on necrosis (%) was also found to be significant with the lowest necrosis induced by employing G_1 and M_2 followed by G_2 and M_2. With regard to overall influence of scion length, grafting technique and media composition on necrosis, L_2, G_1 and M_4; L_3, G_1 and M_2; L_3, G_1 and M_4 resulted in the lowest necrosis of grafted plantlets.

Vitrification percentage was significantly affected (Plate VII.C) by various treatments individually as well in interactive manner (Table 16d). L_2 (5-10 mm) scions resulted in the lowest vitrified plantlets followed by L_3 (10-15 mm) and L_1 (<5 mm) scions. Similarly G_1 (wedge grafting) resulted in significantly lower vitrification as compared to G_2 (horizontal grafting). Regarding media composition, M_3 (MS medium containing 40 g/l sucrose) was statistically better in terms of inducing least vitrification of grafted plantlets.

Combined influence of scion length × grafting technique as shown in Table 16d on per cent vitrification is significant. Minimum per cent vitrification was observed by using L_2 and G_1, which is significantly better than rest of treatment combinations of scion length and grafting technique. Similarly, combined effect of scion length × media on occurrence of vitrification in grafted plantlets is also significant. In this case, L_2 and M_3 proved to be the best combination followed by treatment combination of L_3 and M_3. With regard to combined effect of grafting technique and media, G_1 and M_3 proved to be the best in terms of causing least vitrification (%) in grafted plantlets as compared to rest of treatment combinations.

Data in Table 16e clearly reveals that scion length, grafting technique and medium composition do have an effect on vigour of grafted plantlets under forced scion (S_2).

Statistically vigorous grafted plantlets resulted by using L_2 (5-10 mm scions) as compared to L_1 (<5 mm) and L_3 (10-15 mm) scions. Vigour was observed to be less in G_2 (horizontal grafting) grafted plantlets as compared to G_1 (wedge grafting) grafted plantlets. While finding individual effect of media irrespective of scion length and grafting technique, M_1 (MS medium containing 20 g/l sucrose) and M_4 (MS liquid medium containing 40 g/l sucrose) induced similar vigour while as M_3 (MS medium containing 40 g/l sucrose) resulted in least vigorous plantlets.

The results of combined effect of scion length × grafting technique on vigour are significant (Table 16e). Highly vigorous plantlets resulted by employing treatment combination of L_2 and G_1 followed by $L_1$and G_1. Whereas, combined effect of scion length × media revealed that L_2 and M_4 produced significantly vigorous plantlets, followed by treatment combination of L_2 and M_2. Similarly combined effect of grafting technique × medium composition does have significant effect on vigour of grafted plantlets. Vigorous grafted plantlets were observed by employing G_1 and M_4. Interactive effect of scion length, grafting technique and medium composition on vigour revealed that treatment combination of L_2, G_1 and M_4 resulted in statistically vigorous grafted plantlets (Plate VII.D) as compared to other treatment combinations.

4.2.3 Effect of pre-treatment of growth regulators to scion on graft success, aseptic cultures, necrosis and vitrification.

Two treatment combinations i.e. the treatment showing maximum graft success ($L_2G_1M_2$ using *in vitro* scions, S_1) and minimum graft success ($L_3G_2M_4$ using forced scion, S_2) from previous experiment (Table 15b and Table 16b) were chosen for further study. Microscions were pre-treated with different levels of growth regulators, Thiadiazuron (TDZ) (0.25, 0.50 and 0.75 mgl^{-1}) and 2, 4 dichlorophenoxy acetic acid (2, 4-D) (5.0, 7.5 and 10.0 mgl^{-1}) and their combinations. These microscions were dipped for one minute before carrying grafting using variables as mentioned in above two treatment combinations. Aseptic cultures (%), Graft success (%), necrosis (%) and vitrification (%) of two above treatment combinations during preceding experiment was taken as control.

Application of growth regulator pre-treatment as revealed from Table 17a affected aseptic culture (%), graft success (%), necrosis (%) and vitrification (%) significantly. The perusal of data in Table 17a reveals that per cent aseptic culture significantly increased over control with pre-treatment of TDZ (0.75 mgl^{-1}) + 2, 4-D (5.0 mgl^{-1}) i.e. T_{13} (88.78 %), which is statistically at par with T_2, T_3, T_{10}, T_{11}, T_{14} in $S_1L_2G_1M_2$. While in $S_2L_3G_2M_4$, per cent aseptic cultures increased over control with pre-treatment of TDZ (0.75 mgl^{-1}) i.e. T_3 to 65.32, which is statistically at par with T_2 and T_{13}. Data from table 17a clearly reveals that graft success also increased with pre-treatment of growth regulators (Plate VIII. A, B, C and D). The highest graft success (42.34 %) in $S_1L_2G_1M_2$ was achieved using TDZ (0.5 mgl^{-1}) + 2, 4-

D (7.5 mgl^{-1}) i.e. T_{11}, which is statistically at par with T_8, T_{10} and T_{13}. While, the highest graft success (%) in $S_2L_3G_2M_4$ was also observed by pre-treatment of TDZ (0.5 mgl^{-1}) + 2, 4-D (7.5 mgl^{-1}) i.e. T_{11}, which is statistically non-significant with T_{10}, T_{12}, T_{13}, T_{14} and T_{15}.

From the perusal of data from Table 17b, it can be clearly concluded that pre-treatment of 5 -10 mm *in vitro* or forced scions by TDZ (0.75mgl^{-1}) i.e. T_3 resulted in least necrosis. Likewise, minimum vitrification of micrografts using $S_1L_2G_1M_2$ was observed when microscions were pre-treated with TDZ (0.75 mgl^{-1}) + 2,4-D (7.5 mgl^{-1}) i.e. T_{14}, which is statistically at par with T_{15}. While as minimum per cent vitrified micrografts in $S_2L_3G_2M_4$ was observed with pre-treatment of TDZ (0.75 mgl^{-1}) + 2, 4-D (5.0 mgl^{-1}) i.e. T_{13}. This treatment is statistically at par in terms of inducing less vitrification with T_{10}, T_{11}, and T_{14} (Table 17b).

Bhatt (2008) also reported beneficial effects of pre treatment of scion by growth regulators and antioxidants on graft success, asepsis and vitrification. He also reported that use of pre-treated apices not only markedly increase the grafting success but also overcome problems encountered during the handling of *in vitro* micrografting that results in browning and drying of apices. Together with auxins, cytokinins stimulate both apex initiation and the union between rootstock and scion by increasing vascular bundle formation (Quoirin *et al* 1977). Similarly Rafail and Mosleh (2010) obtained higher success rate in apple and pear micrografting by using cytokinins and confirmed the value cytokinins in promoting and inducing callus growth and the formation of graft union between rootstocks and scions. Cytokinins rejuvenate plant cells and stimulate cell proliferation of graft union tissue with auxins (Bessis 1986). Jeffree and Yeoman (1983) reported that application of growth regulators at the point of graft union increases the potential regenerative tissue, allowing the formation of new cellular layer, assuring the establishment of connectivity of vascular tissues between the two graft partners. Parthasarathy *et al* (1997) reported that immersion of Khasi mandarin shoot tips in 2,4-D (10µg l^{-1}) for 5 minutes before grafting improved the grafting success and achieved good graft union and complete vascular connection when these shoot tips were grafted on Cleopatra mandarin, Rangpur lime, Kagzi lime and Khasi mandarin. Starrantino and Caruso (1988) found that the use of growth regulator such as BAP increased the potential regenerative tissue and contributed to increase the rate of graft success in citrus from 73 to 91 per cent. Similarly, Nunes *et al* (2005) found that the IBA, associated with MS medium applied at the point of grafting, promoted further development of micrograft in apple. Shide *et al* (2008) also reported pre-treatment of shoot tips with BAP (0.5 mgl^{-1}) for 10 min before grafting significantly increased the grafting success of micrografted plant followed by 2, 4-D (10 mgl^{-1}). Similar beneficial effect of treating the apices with growth regulators were observed by Edriss and Burger (1984), Starrantino *et al* (1986), Kumarin *et al* (2000), Lahoty *et al* (2013) and Hoa *et al* (2004) in different fruit crops. However, the highest concentration of these growth regulators decreased the success rate and increased the browning/necrosis

incidence. These higher concentrations might have affected the membrane electric properties, charge and thus increased ion leakage. Similar results were reported by Filek *et al* (2002) in winter wheat cells and Moghadam *et al* (2012) in cactus.

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CHAPTER V

SUMMARY

The present investigation "*In vitro* propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burm f.) Nakai) pear" was carried out in Tissue Culture Laboratory in the Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2011-13. The whole investigation comprised of four experiments. The results obtained during the course of study are summarized as under:

- ***In vitro* propagation of Patharnakh and Kainth**
  - Murashige and Skoog medium (M_2) fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) resulted in least necrotic cultures (0.00 %) in Patharnakh (forced) however, M_2 fortified with BAP (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) resulted in statistically minimum necrotic cultures (2.04 %) in Patharnakh (active).
  - In Kainth (forced) and Kainth (active), minimum necrotic culture of 3.05 and 2.10 % respectively resulted by using M_2 medium fortified with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}).
  - Establishment of Patharnakh (forced) was maximum (96.10 %) in Woody Plant Medium (M_3) supplemented with BAP (1.5 mgl^{-1}) and it was at par with M_2 supplemented with BAP (1.5 mgl^{-1}) and IBA (0.5 mgl^{-1}) and M_2 with BAP (1.5 mgl^{-1}) and IBA (0.01 mgl^{-1}), while as M_2 medium fortified with BAP (1.5 mgl^{-1}) + IBA (0.01 mgl^{-1}) resulted in statistically maximum establishment (96.29 %) in Patharnakh (active).
  - Murashige and Skoog medium (M_2) supplemented with BAP (1.5 mgl^{-1}) and IBA (0.25 mgl^{-1}) resulted in maximum culture establishment of 52.80 and 63.60 % respectively in Kainth (forced) and Kainth (active).
  - There was non-significant interaction between type of medium and BAP concentration in Patharnakh (forced) in terms of proliferated cultures, although the highest proliferated cultures (85.67 %) was obtained in Woody Plant Medium (M_3) fortified with BAP (2.5 mgl^{-1}). M_3 containing BAP (5.0 mgl^{-1}) resulted in significantly highest proliferated cultures (79.47 %) in Patharnakh (active).
  - Maximum proliferation rate, i.e. shoots per explant (3.80) was produced in M_3 medium supplemented with BAP (2.5 mgl^{-1}) in Patharnakh (forced) whereas, M_2 fortified with BAP (2.5 mgl^{-1}) resulted in maximum number of shoots per explant (3.08) followed by 2.84 in M_3 supplemented with BAP (5.0 mgl^{-1}) in Patharnakh (active).

- Shoots of maximum average length in Patharnakh (forced), i.e. 53.75 mm and Patharnakh (active) i.e. 51.33 mm were obtained by using M_3 fortified with BAP (0.5 mgl^{-1}) and BAP (0.00 mgl^{-1}) respectively.
- Maximum proliferation of 95.30 and 83.19 % were obtained by using M_3 fortified with BAP (3.0 mgl^{-1}) in Kainth (forced) and Kainth (active) respectively.
- In Kainth (forced) and Kainth (active), M_3 medium fortified with BAP (3.0 mgl^{-1}) resulted in maximum number of shoots per explant, i.e. 6.28 and 5.93 respectively.
- There was no significant interaction between type of media and growth regulator level on average shoot length produced in Kainth (forced) although maximum value (44.20 mm) was obtained when basal M_3 medium, i.e. control was used during shoot proliferation. However, in Kainth (active) statistically longest shoots (46.32 mm) were obtained in M_3 medium that was free of BAP followed by M_3 fortified with BAP (0.5 mgl^{-1}).
- The highest rooting response (10.16 %) in Patharnakh (forced) was obtained in half-strength Murashige and Skoog medium (M_1) fortified with IBA (1.0 mgl^{-1}). However, rooting of 9.16 % resulted in M_1 medium supplemented with IBA (1.0 mgl^{-1}).
- Half strength Murashige and Skoog medium (M_1) fortified with IBA (1.0 mgl^{-1}) resulted in maximum number of roots per explant in Patharnakh (forced) i.e. 2.38 and Patharnakh (active) i.e. 2.60.
- Root length was also observed to be significantly affected by medium and growth regulator fortification. Woody Plant medium (M_3) supplemented with IBA (1.0 mgl^{-1}) resulted in maximum average root length of 28.57 mm in Patharnakh (forced) and 33.13 mm in Patharnakh (active).
- No rooting was observed in Patharnakh (forced) and Patharnakh (active) irrespective of media and various levels of NAA used.
- In Kainth (forced) and Kainth (forced), maximum rooting of 13.34 and 14.08 % respectively were obtained in M_1 fortified with IBA (0.1 mgl^{-1}).
- M_1 medium fortified with IBA (1.0 mgl^{-1}) resulted in significantly higher number of roots per explant, i.e. 2.79 and 2.60 respectively in Kainth (forced) and Kainth (active).
- Woody Plant medium (M_3) fortified with IBA (0.1 mgl^{-1}) produced statistically maximum root length of 31.15 mm in Kainth (forced) and 31.10 mm in Kainth (active).
- As compared to IBA, NAA induced higher rooting in both Kainth (forced) and Kainth (active) irrespective of type of rooting media used.

- Half-strength Murashige and Skoog medium (M_1) fortified with NAA (1.0 mgl^{-1}) resulted in maximum rooting of 29.61 % in Kainth (forced) and 27.46 % in Kainth (active).
- Highest number of roots per explant (3.40) in Kainth (forced) were produced in M_1 medium fortified with NAA (1.0 mgl^{-1}) whereas, M_2 medium fortification with NAA (1.0 mgl^{-1}) resulted in maximum number of roots per explant (3.26) in Kainth (active).
- Similarly, average root length was also observed to be significantly affected by medium and NAA concentration. In Kainth (forced), a treatment combination of M_3 fortified with NAA (0.1 mgl^{-1}) resulted in maximum average length of roots (22.97 mm). Whereas, in Kainth (active), M_3 medium supplemented with NAA (1.0 mgl^{-1}) resulted in longer roots (23.22 mm) followed by 21.56 mm in M_3 medium fortified with NAA (0.1 mgl^{-1}).
- *In vitro* derived Patharnakh scion resulted in higher per cent graft success, higher aseptic culture percentage, lower necrosis and less vitrification of grafted plantlets as compared to *in vivo* derived Patharnakh scions.
- Higher aseptic cultures (84.51 %) using *in vitro* scion (S_1) resulted by using 5-10 mm (L_2) scion grafted by horizontal grafting (G_2) allowed to grow in Murashige and Skoog (MS) liquid medium containing 20 g/l sucrose (M_2), while as higher per cent aseptic culture (67.21 %) using *in vivo* scion (S_2) resulted by using 5-10 mm (L_2) employing wedge grafting (G_1) allowed to grow in Murashige and Skoog (MS) medium containing 20 g/l sucrose (M_1).
- Highest graft success using *in vitro* scion (33.71 %) and *in vivo* scion (18.55 %) resulted by using 5-10 mm (L_2) size scions employing wedge grafting (G_1) and allowed to grow in Murashige and Skoog (MS) liquid medium containing 20 g/l sucrose (M_2).
- Necrosis percentage of grafted plantlets using *in vitro* scion was lower by employing 10-15 mm (L_2) scions grafted by wedge grafting (G_1) grown in MS liquid medium containing 40g/l sucrose (M_4); less than 5 mm (L_1) scions grafted using horizontal grafting (G_2) grown in MS liquid medium containing 20 g/l sucrose (M_2); less than 5 mm (L_1) scions grafted using horizontal grafting (G_2) grown in MS liquid medium containing 40 g/l sucrose (M_4) and 5-10 mm (L_2) scions grafted using horizontal grafting (G_2) grown in MS liquid medium containing 40 g/l sucrose (M_4). These treatment combinations were statistically at par in terms of inducing least necrosis in grafted plantlets. While as per cent necrosis using *in vivo* scions was lowest (0.00 %) by using L_2, G_1 and M_4; L_3 (10-15 mm), G_1 and M_2; L_3, G_1 and M_4.

- Least percentage of vitrified grafted plantlets using *in vitro* scions i.e. 10.41 % were obtained by using L_2 size scion employing wedge grafting raised in Murashige and Skoog (MS) medium containing 40 g/l sucrose. Similarly, minimum vitrification (15.19 %) resulted by using L_2 size scions grafted by wedge grafting and allowed to grow in M_3, in case of *in vivo* scion source.
- Highly vigorous plantlets were produced by using L_2 (5-10 mm), G_1 (wedge grafting) and M_2 (MS liquid medium containing 20g/l sucrose) followed by those plantlets employing L_2, G_1 and M_3 (Murashige and Skoog (MS) medium containing 40 g/l sucrose), in case of *in vitro* scion source. However, in case of using *in vivo* scion source, statistically vigorous grafted plantlets resulted by employing L_2, G_1 and M_4 as compared to other treatment combinations.
- Highest aseptic cultures (88.78 %) resulted by pre-treatment of TDZ (0.75 mgl^{-1}) + 2, 4-D (5.0 mgl^{-1}) while using *in vitro* scion. However, using *in vivo* scions maximum aseptic cultures (65.32 %) resulted with pre-treatment of TDZ (0.75 mgl^{-1}).
- Graft success reached 42.34 per cent using TDZ (0.5 mgl^{-1}) + 2, 4-D (7.5 mgl^{-1}) pre-treatment in case of employing *in vitro* derived scion which was significantly better than control. Whereas, using *in vivo* scion, maximum graft success (19.87 %) resulted by pre-treatment with TDZ (0.5 mgl^{-1}) + 2, 4-D (7.5 mgl^{-1}).
- Pre-treatment of both *in vitro* and *in vivo* scions with TDZ (0.75 mgl^{-1}) resulted in least necrosis of grafted plantlets.
- Minimum vitrification (4.11 %) of micrografts using *in vitro* scion was observed when microscions were pre-treated with TDZ (0.75 mgl^{-1}) + 2, 4-D (7.5 mgl^{-1}). However, minimum vitrified micrografts (28.86 %) using *in vivo* scion resulted with pre-treatment of TDZ (0.75 mgl^{-1}) + 2, 4-D (5.0 mgl^{-1}).

Appendix-I

Complete Data Tables and plates

Table 1a: Effect of media and growth regulators (mgl^{-1}) on per cent necrosis in Patharnakh (forced) explants during establishment stage

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	9.02	6.10	9.44	8.19
BAP(0.5)+IBA(0.25)	4.29	3.25	14.18	7.24
BAP(0.5)+IBA(0.5)	8.99	0.57	19.72	9.76
BAP(1.0)+IBA(0.01)	12.38	0.00	0.67	4.35
BAP(1.0)+IBA(0.25)	3.54	3.02	8.38	4.98
BAP(1.0)+IBA(0.5)	5.33	4.53	8.49	6.12
BAP(1.5)+IBA(0.01)	7.22	4.94	8.55	6.90
BAP(1.5)+IBA(0.25)	9.04	4.24	9.30	7.53
BAP(1.5)+IBA(0.5)	12.49	6.79	3.84	7.71
BAP (0.0)+IBA(0.0)	16.83	8.12	14.33	13.09
Mean	8.91	4.16	9.69	
C.D($p \leq 0.05$)	Medium (A)=0.464, GR (B)=0.930, A×B=1.483			

Table 1b: Effect of media and growth regulators (mgl^{-1}) on per cent necrosis in Patharnakh (active) explants during establishment stage

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	23.50	14.12	19.41	19.01
BAP(0.5)+IBA(0.25)	19.39	8.19	16.15	14.58
BAP(0.5)+IBA(0.5)	25.06	6.00	15.28	15.45
BAP(1.0)+IBA(0.01)	16.16	2.04	17.08	11.76
BAP(1.0)+IBA(0.25)	19.31	16.15	18.62	18.03
BAP(1.0)+IBA(0.5)	15.69	8.64	12.25	12.19
BAP(1.5)+IBA(0.01)	12.67	16.83	17.21	15.57
BAP(1.5)+IBA(0.25)	17.65	11.49	14.47	14.54
BAP(1.5)+IBA(0.5)	27.00	14.05	18.50	19.85
BAP (0.0)+IBA(0.0)	24.53	8.30	16.93	16.59
Mean	20.10	10.58	16.59	
C.D($p \leq 0.05$)	Medium (A)=0.475, GR (B)=1.132, A×B=1.933			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine
IBA Indole acetic acid

Table 2a: Effect of media type and growth regulators (mgl^{-1}) on per cent necrosis in Kainth (forced) explants during establishment stage

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	12.56	8.33	8.62	9.84
BAP(0.5)+IBA(1.0)	18.19	16.15	18.51	17.62
BAP(0.5)+IBA(2.0)	22.29	23.26	18.94	21.50
BAP(1.5)+IBA(0.01)	7.94	3.05	6.50	5.83
BAP(1.5)+IBA(1.0)	16.37	7.10	13.45	12.31
BAP(1.5)+IBA(2.0)	19.84	16.34	18.33	18.17
BAP(3.0)+IBA(0.01)	9.50	10.44	6.71	8.88
BAP(3.0)+IBA(1.0)	19.56	17.02	18.16	18.25
BAP(3.0)+IBA(2.0)	27.05	22.27	24.30	24.54
BAP (0.0)+IBA(0.0)	10.08	7.67	9.86	9.20
Mean	16.34	13.16	14.34	
C.D ($p \leq 0.05$)	**Medium (A)=0.453, GR (B)=1.098, A×B=1.649**			

Table 2b: Effect of media type and growth regulators (mgl^{-1}) on per cent necrosis in Kainth (active) explants during establishment stage

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	15.48	7.79	8.69	10.65
BAP(0.5)+IBA(1.0)	25.23	22.17	10.35	19.25
BAP(0.5)+IBA(2.0)	28.66	27.52	10.80	22.33
BAP(1.5)+IBA(0.01)	4.71	2.10	12.25	6.35
BAP(1.5)+IBA(1.0)	17.25	7.11	23.17	15.84
BAP(1.5)+IBA(2.0)	15.16	9.48	15.47	13.37
BAP(3.0)+IBA(0.01)	6.62	3.50	17.22	9.11
BAP(3.0)+IBA(1.0)	10.25	4.27	20.16	11.56
BAP(3.0)+IBA(2.0)	8.05	14.33	17.52	13.30
BAP (0.0)+IBA(0.0)	16.45	14.89	14.19	15.18
Mean	14.79	11.32	14.98	
C.D($p \leq 0.05$)	**Medium (A)=0.342, GR (B)=0.706, A×B=1.296**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine
IBA Indole acetic acid

Table 3a: Effect of media type and growth regulators (mg/l) on per cent establishment of Patharnakh (forced) explant

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	72.43	62.57	40.20	58.40
BAP(0.5)+IBA(0.25)	80.79	87.56	40.10	69.48
BAP(0.5)+IBA(0.5)	58.74	52.19	54.61	55.18
BAP(1.0)+IBA(0.01)	59.87	70.03	62.08	63.99
BAP(1.0)+IBA(0.25)	84.23	67.99	73.52	75.25
BAP(1.0)+IBA(0.5)	78.51	47.78	52.55	59.61
BAP(1.5)+IBA(0.01)	59.51	95.39	96.10	83.67
BAP(1.5)+IBA(0.25)	88.20	80.26	66.31	78.26
BAP(1.5)+IBA(0.5)	63.80	95.52	63.60	74.31
BAP (0.0)+IBA(0.0)	31.86	37.53	25.58	31.66
Mean	67.79	69.68	57.47	
C.D ($p \leq 0.05$)	**Medium (A)=0.996, GR (B)= 1.818, A×B=3.150**			

Table 3b: Effect of media type and growth regulators (mg/l) on per cent establishment of Patharnakh (active) explant

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	28.18	75.20	18.72	40.70
BAP(0.5)+IBA(0.25)	38.20	57.45	18.74	38.13
BAP(0.5)+IBA(0.5)	23.56	59.43	14.40	32.46
BAP(1.0)+IBA(0.01)	26.37	70.71	42.81	46.63
BAP(1.0)+IBA(0.25)	0.76	18.34	8.30	9.13
BAP(1.0)+IBA(0.5)	9.11	42.14	25.07	25.44
BAP(1.5)+IBA(0.01)	63.7	96.29	90.67	83.55
BAP(1.5)+IBA(0.25)	52.16	52.78	73.46	59.47
BAP(1.5)+IBA(0.5)	34.65	38.73	66.52	46.63
BAP (0.0)+IBA(0.0)	9.18	15.44	6.16	10.26
Mean	28.59	52.65	36.49	
C.D ($p \leq 0.05$)	**Medium (A)=0.852, GR (B)= 1.556, A×B=2.695**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine
IBA Indole acetic acid

Table 4a: Effect of media type and growth regulators (mg/l) on per cent explant establishment in Kainth (forced)

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	21.32	25.05	16.61	20.99
BAP(0.5)+IBA(0.25)	0.40	9.11	25.04	11.52
BAP(0.5)+IBA(0.5)	0.05	0.21	12.47	4.24
BAP(1.0)+IBA(0.01)	11.12	26.68	50.14	29.31
BAP(1.0)+IBA(0.25)	8.36	14.19	41.49	21.35
BAP(1.0)+IBA(0.5)	5.29	11.29	11.72	9.43
BAP(1.5)+IBA(0.01)	18.82	26.36	31.27	25.48
BAP(1.5)+IBA(0.25)	11.06	52.80	27.30	30.39
BAP(1.5)+IBA(0.5)	5.09	18.71	14.30	12.70
BAP (0.0)+IBA(0.0)	5.67	10.40	5.12	7.063
Mean	8.72	19.48	23.55	
C.D ($p \leq 0.05$)	**Medium (A)=0.881, GR (B)= 1.608, A×B=2.786**			

Table 4b: Effect of media type and growth regulators (mg/l) on per cent explant establishment in Kainth (active)

Growth regulator combination (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)+IBA(0.01)	36.80	23.08	18.25	26.04
BAP(0.5)+IBA(0.25)	28.63	25.11	20.02	24.59
BAP(0.5)+IBA(0.5)	12.54	21.88	18.21	17.54
BAP(1.0)+IBA(0.01)	30.86	36.40	36.34	34.53
BAP(1.0)+IBA(0.25)	40.01	36.37	23.07	33.15
BAP(1.0)+IBA(0.5)	19.98	30.75	38.45	29.73
BAP(1.5)+IBA(0.01)	37.58	50.42	18.60	35.53
BAP(1.5)+IBA(0.25)	46.62	63.60	47.38	52.53
BAP(1.5)+IBA(0.5)	15.77	14.31	18.68	16.25
BAP (0.0)+IBA(0.0)	13.40	19.45	10.68	14.51
Mean	28.22	32.14	24.97	
C.D ($p \leq 0.05$)	**Medium (A)=1.001, GR (B)= 1.827, A×B=3.165**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine
IBA Indole acetic acid

Table 5a: Effect of media type and growth regulator level (mgl^{-1}) on per cent proliferated cultures in Patharnakh (forced)

Growth regulator (mgl^{-1})	Media			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	46.30	66.57	68.68	60.52
BAP(2.5)	49.71	54.34	85.67	63.24
BAP(5.0)	60.00	71.72	58.38	63.37
BAP(0.0)	22.56	26.50	40.03	29.70
Mean	44.64	54.78	63.19	
C.D ($p \leq 0.05$)	**Medium (A)= 9.854, BAP (B)= 11.378, A×B=NS**			

Table 5b: Effect of media type and growth regulator level (mgl^{-1}) on number of shoots produced per explant in Patharnakh (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	3.00	2.77	3.26	3.0
BAP(2.5)	3.66	3.22	3.80	3.6
BAP(5.0)	2.83	3.00	2.67	2.8
BAP(0.0)	1.20	1.41	1.65	1.4
Mean	2.67	2.60	2.85	
C.D ($p \leq 0.05$)	**Medium (A)= 0.160, BAP(B)= 0.185, A×B=0.320**			

Table 5c: Effect of media type and growth regulator level (mgl^{-1}) on average length of shoots (mm) in Patharnakh (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	50.05	44.53	53.75	49.44
BAP(2.5)	46.20	33.58	49.85	43.21
BAP(5.0)	40.43	25.30	46.33	37.35
BAP(0.0)	50.18	43.91	48.92	47.67
Mean	46.72	36.83	49.71	
C.D ($p \leq 0.05$)	**Medium (A)= 1.992, BAP (B)= 2.301, A×B=3.984**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine

Table 6a: Effect of media type and growth regulator level (mgl^{-1}) on per cent proliferated cultures in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	55.47	48.39	66.51	56.79
BAP(2.5)	62.16	59.28	68.48	63.31
BAP(5.0)	65.78	69.62	79.47	71.62
BAP(0.0)	21.01	27.82	12.92	20.58
Mean	51.11	51.28	56.85	
C.D ($p \leq 0.05$)	**Medium (A)= 2.620, BAP (B)= 3.025, A×B=5.240**			

Table 6b: Effect of media type and growth regulator level (mgl^{-1}) on number of shoots produced per explant in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	2.30	2.46	2.75	2.50
BAP(2.5)	2.69	3.08	2.80	2.86
BAP(5.0)	2.46	2.51	2.84	2.60
BAP(0.0)	1.09	1.47	1.73	1.43
Mean	2.14	2.38	2.53	
C.D ($p \leq 0.05$)	**Medium (A)= 0.168, BAP (B)= 0.194, A×B=NS**			

Table 6c: Effect of media type and growth regulator level (mgl^{-1}) on average length of shoots (mm) in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	43.13	42.48	42.78	42.80
BAP(2.5)	39.55	31.10	42.22	37.62
BAP(5.0)	36.18	20.13	41.38	32.56
BAP(0.0)	43.39	40.40	51.33	45.04
Mean	40.56	33.53	44.43	
C.D ($p \leq 0.05$)	**Medium (A)= 2.126, BAP (B)= 2.155, A×B=4.052**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine

Table 7a: Effect of media type and growth regulator level (mgl^{-1}) on proliferated culture percentage in Kainth (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	60.03	71.43	66.65	66.04
BAP(1.5)	84.59	92.51	92.35	89.82
BAP(3.0)	86.44	91.64	95.30	91.13
BAP(0.0)	13.93	25.49	34.23	24.55
Mean	61.25	70.27	72.13	
C.D (p≤0.05)	**Medium (A)= 1.551, BAP (B)= 1.790, A×B=3.101**			

Table 7b: Effect of media type and growth regulator level (mgl^{-1}) on number of shoots produced per explant in Kainth (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	2.76	2.59	2.91	2.75
BAP(1.5)	2.92	4.61	5.75	4.43
BAP(3.0)	3.15	4.92	6.28	4.78
BAP(0.0)	1.68	2.33	2.62	2.21
Mean	2.63	3.61	4.39	
C.D (p≤0.05)	**Medium (A)= 0.141, BAP (B)= 0.163, A×B=0.282**			

Table 7c: Effect of media type and growth regulator level (mgl^{-1}) on average length of shoots (mm) in Kainth (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP(0.5)	42.10	40.31	42.97	41.79
BAP(1.5)	39.05	33.53	39.01	37.20
BAP(3.0)	31.02	31.37	37.26	33.22
BAP(0.0)	43.27	39.11	44.20	42.19
Mean	38.86	36.08	40.86	
C.D (p≤0.05)	**Medium (A)= 1.924, BAP (B)= 2.222, A×B=NS**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine

Table 8a: Effect of media type and growth regulator level (mgl^{-1}) on proliferated culture percentage in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP (0.5)	63.25	36.63	50.49	50.12
BAP (1.5)	45.12	52.92	73.32	57.12
BAP (3.0)	70.79	40.38	83.19	64.79
BAP(0.0)	11.73	14.35	29.36	18.48
Mean	47.72	36.07	59.09	
C.D ($p \leq 0.05$)	**Medium (A)= 2.352, BAP (B)= 2.716, A×B=4.705**			

Table 8b: Effect of media type and growth regulator level (mgl^{-1}) on number of shoots produced per explant in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP (0.5)	2.33	3.70	2.81	2.95
BAP (1.5)	2.90	4.23	5.13	4.09
BAP (3.0)	3.28	4.74	5.93	4.65
BAP(0.0)	1.54	2.23	2.51	2.09
Mean	2.51	3.73	4.10	
C.D ($p \leq 0.05$)	**Medium (A)= 0.126, BAP (B)= 0.145 , A×B=0.251**			

Table 8c: Effect of media type and growth regulator level (mgl^{-1}) on average length of shoots (mm) in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
BAP (0.5)	40.65	40.35	43.40	41.47
BAP (1.5)	37.77	35.18	41.00	37.98
BAP (3.0)	28.25	32.22	36.97	32.48
BAP(0.0)	37.23	36.35	46.32	39.97
Mean	35.98	36.03	41.92	
C.D ($p \leq 0.05$)	**Medium (A)= 1.582, BAP (B)= 1.826, A×B=3.163**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
BAP Benzyl amino purine

Table 9a: Effect of media type and IBA level (mgl^{-1}) on rooting per cent in Patharnakh (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	10.16	8.48	5.74	8.13
IBA (1.5)	7.58	0.00	0.00	2.53
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	3.55	1.70	1.15	
C.D (p≤0.05)	**Medium (A)= 0.302, IBA(B)= 0.389, A×B=0.674**			

Table 9b: Effect of media type and IBA level (mgl^{-1}) on number of roots per explant in Patharnakh (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	2.38	1.40	1.19	1.66
IBA (1.5)	1.60	0.00	0.00	0.53
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	0.80	0.28	0.24	
C.D (p≤0.05)	**Medium (A)= 0.052, IBA(B)= 0.067, A×B=0.116**			

Table 9c: Effect of media type and IBA level (mgl^{-1}) on average length of roots (mm) in Patharnakh (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	15.90	24.00	28.57	22.82
IBA (1.5)	22.07	0.00	0.00	7.36
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	7.59	4.80	5.71	
C.D (p≤0.05)	**Medium (A)= 0.464, IBA(B)= 0.598, A×B=1.037**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
IBA Indole butyric acid

Table 10a: Effect of media type and IBA level (mgl^{-1}) on rooting per cent in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	9.16	5.22	4.82	6.40
IBA (1.5)	6.26	0.00	0.00	2.09
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	3.08	1.04	0.96	
C.D (p≤0.05)	**Medium (A)= 0.201, IBA(B)= 0.258, A×B=0.446**			

Table 10b: Effect of media type and IBA level (mgl^{-1}) on number of roots per explant in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	2.60	1.80	1.41	1.94
IBA (1.5)	1.68	0.00	0.00	0.56
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	0.86	0.36	0.28	
C.D (p≤0.05)	**Medium (A)= 0.034, IBA(B)= 0.438, A×B=0.758**			

Table 10c: Effect of media type and IBA level (mgl^{-1}) on average length of roots (mm) in Patharnakh (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.5)	0.00	0.00	0.00	0.00
IBA (1.0)	14.22	25.20	33.13	24.18
IBA (1.5)	20.96	0.00	0.00	6.99
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	7.04	5.04	6.63	
C.D (p≤0.05)	**Media (A)= 0.524, IBA(B)= 0.676, A×B=1.171**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
IBA Indole butyric acid

Table 11a: Effect of media and IBA (mgl^{-1}) on per cent rooting in Kainth (forced) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.1)	13.34	9.04	9.36	10.58
IBA (1.0)	7.55	0.00	0.00	2.52
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	5.22	2.26	2.34	
C.D ($p \leq 0.05$)	**Medium (A)= 0.705, IBA(B)= 0.815, A×B=1.411**			

Table 11b: Effect of media and IBA (mgl^{-1}) on number of roots per explant in Kainth (forced)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.1)	2.20	2.01	1.81	2.01
IBA (1.0)	2.79	0.00	0.00	0.93
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	1.25	0.50	0.45	
C.D ($p \leq 0.05$)	**Medium (A)= 0.057, IBA(B)= .066, A×B=.114**			

Table 11c: Effect of media and IBA (mgl^{-1}) on average length of roots (mm) in Kainth (forced) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
IBA (0.1)	22.90	28.17	31.15	27.41
IBA (1.0)	21.10	0.00	0.00	7.03
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	11.00	7.04	7.79	
C.D($p \leq 0.05$)	**Medium (A)= 1.226, IBA(B)= 1.416, A×B=2.452**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
IBA Indole butyric acid

Table 12a: Effect of media type and NAA level (mgl^{-1}) on rooting (%) in Kainth (forced) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	24.35	23.41	19.30	22.35
NAA (1.0)	29.61	24.94	19.95	24.83
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	13.49	12.09	9.81	
C.D (p≤0.05)	**Medium (A)=1.006, NAA(B)=1.162, A×B=2.012**			

Table 12b: Effect of media and NAA (mgl^{-1}) on number of roots per explant in Kainth (forced) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	3.02	2.78	2.43	2.74
NAA (1.0)	3.40	3.22	2.60	3.07
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	1.61	1.50	1.26	
C.D (p≤0.05)	**Medium (A)= 0.057, NAA(B)= 0.065, A×B=0.113**			

Table 12c: Effect of media and NAA (mgl^{-1}) on average length of roots (mm) in Kainth (forced) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1(1/2 MS)	M_2(MS)	M_3(WPM)	
NAA (0.1)	18.23	20.40	22.97	20.53
NAA (1.0)	14.33	15.37	21.20	16.97
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	8.14	8.94	11.04	
C.D (p≤0.05)	**Medium (A)= 0.919, NAA(B)= 1.061, A×B=1.837**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
NAA Naphthalene acetic acid

Table 13a: Effect of media and IBA (mgl^{-1}) on per cent rooting in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.1)	14.08	8.90	8.39	10.46
IBA (1.0)	8.67	0.00	0.00	2.89
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	5.69	2.23	2.10	
C.D (p≤0.05)	**Medium (A)= 0.814, IBA(B)=0.940, A×B=1.628**			

Table 13b: Effect of media and IBA (mgl^{-1}) on number of roots per explant in Kainth (active) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.1)	1.98	1.86	1.65	1.83
IBA (1.0)	2.60	0.00	0.00	0.87
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	1.15	0.47	0.41	
C.D (p≤0.05)	**Media (A)= 0.053, IBA(B)= 0.061, A×B=0.106**			

Table 13c: Effect of media and IBA (mgl^{-1}) on average length of roots (mm) in Kainth (active) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
IBA (0.1)	23.30	29.00	31.10	27.80
IBA (1.0)	19.60	0.00	0.00	6.53
IBA (2.0)	0.00	0.00	0.00	0.00
IBA (0.0)	0.00	0.00	0.00	0.00
Mean	10.73	7.25	7.78	
C.D (p≤0.05)	**Medium (A) = 0.856, IBA (B) = 0.988. A×B=1.711**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
IBA Indole butyric acid

Table 14a: Effect of media and NAA (mgl^{-1}) on per cent rooting in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
NAA (0.1)	23.15	22.74	21.63	22.51
NAA (1.0)	27.46	24.58	16.65	22.90
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	12.65	11.83	9.57	
C.D (p≤0.05)	**Medium (A)=0.754, NAA(B)=0.871, A×B=1.508**			

Table 14b: Effect of media and NAA (mgl^{-1}) on number of roots per explant in Kainth (active) micro-shoot

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
NAA (0.1)	2.98	3.02	1.90	2.63
NAA (1.0)	3.26	3.60	1.66	2.84
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	1.56	1.66	0.89	
C.D (p≤0.05)	**Medium (A)= 0.061, NAA(B)= 0.070, A×B=0.121**			

Table 14c: Effect of media and NAA (mgl^{-1}) on average length of roots (mm) in Kainth (active)

Growth regulator (mgl^{-1})	Medium			Mean
	M_1 (1/2 MS)	M_2 (MS)	M_3 (WPM)	
NAA (0.1)	18.97	19.33	21.56	19.95
NAA (1.0)	14.30	14.22	23.22	17.25
NAA (2.0)	0.00	0.00	0.00	0.00
NAA (0.0)	0.00	0.00	0.00	0.00
Mean	8.32	8.39	11.20	
C.D (p≤0.05)	**Medium (A)= 0.705, NAA(B)= 0.814, A×B=1.409**			

M_1 Murashige and Skoog medium containing half-strength of macro and micronutrients
M_2 Full-strength Murashige and Skoog medium
M_3 Woody Plant medium
NAA Naphthalene acetic acid

Table 15a: Effect of scion length, grafting technique and media composition on per cent aseptic cultures using *in vitro* scions (S_1)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		76.35	76.25	75.15	72.63	75.10	78.51	76.64	76.31	74.26	76.43
L_2		80.38	78.68	76.05	76.01	77.78	84.34	81.37	77.55	77.07	80.08
L_3		72.51	72.27	70.55	69.97	71.33	74.45	70.73	70.73	69.16	71.27
Mean		76.41	75.73	73.92	72.87		79.10	76.25	74.86	73.50	
C.D($p \leq 0.05$)		Scion length : 0.328					Scion length × grafting technique : 0.464				
		Grafting technique : 0.268					Scion length × medium : 0.656				
		Medium : 0.379					Grafting technique × medium : 0.536				

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 15b: Effect of scion length, grafting technique and media composition on graft success using *in vitro* scion source (S_1)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		27.09	30.36	28.08	27.36	28.22	21.76	21.49	21.51	19.77	21.13
L_2		30.14	33.71	31.46	32.42	31.93	26.49	26.99	26.08	23.90	25.87
L_3		19.41	21.38	19.85	22.22	20.72	16.67	19.13	18.30	19.07	18.29
Mean		25.55	28.48	26.46	27.33		21.64	22.54	21.96	20.91	
CD ($p \leq 0.05$)		Scion length : 0.862					Scion length × grafting technique : 1.219				
		Grafting technique : 0.704					Scion length × medium : NS				
		Medium : 0.995					Grafting technique × medium : 1.407				

Table 15c: Effect of scion length, grafting technique and media composition on per cent necrosis using *in vitro* scion source (S_1)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		29.60	14.04	32.84	21.64	24.53	17.81	0.00	24.55	0.00	10.59
L_2		16.47	6.71	14.32	12.09	12.40	59.85	33.44	16.21	0.00	27.38
L_3		80.39	48.84	22.26	0.00	37.87	87.07	60.15	74.86	49.55	67.91
Mean		42.15	23.20	23.14	11.24		54.91	31.20	38.54	16.52	
C.D (p≤0.05)		Scion length : 0.765				Scion length × grafting technique : 1.082					
		Grafting technique : 0.625				Scion length × medium : 1.530					
		Medium : 0.884				Grafting technique × medium : 1.249					

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 15d: Effect of scion length, grafting technique and media composition on per cent vitrification using *in vitro* scion source (S_1)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		16.23	19.23	12.51	18.01	16.50	26.67	32.60	22.25	30.41	27.98
L_2		14.05	18.94	10.41	17.53	15.23	21.21	28.59	19.26	29.86	24.73
L_3		21.99	25.33	16.49	22.15	21.49	23.64	29.24	14.37	25.99	23.31
Mean		17.42	21.17	13.14	19.23		23.84	30.14	18.63	28.75	
C.D (p≤0.05)		Scion length : 1.037				Scion length × grafting technique : 1.467					
		Grafting technique : 0.847				Scion length × medium : 2.074					
		Medium : 1.198				Grafting technique × medium : 1.694					

Table 15e: Effect of scion length, grafting technique and media composition on vigour (1-3 point scale) using *in vitro* scion source (S_1)

Scion length	**Grafting technique**	**G_1**				Mean	**G_2**				Mean
	Medium	**M_1**	**M_2**	**M_3**	**M_4**		**M_1**	**M_2**	**M_3**	**M_4**	
L_1		2.38	2.57	2.21	2.52	2.42	1.78	2.27	2.43	2.31	2.20
L_2		2.60	2.68	2.66	2.53	2.62	1.73	1.92	2.01	2.34	2.00
L_3		1.53	1.94	2.04	2.44	1.99	1.06	1.43	1.21	1.60	1.33
Mean		2.17	2.40	2.30	2.50		1.52	1.87	1.88	2.08	
C.D ($p \leq 0.05$)		Scion length : 0.045				Scion length × grafting technique : 0.064					
		Grafting technique : 0.037				Scion length × medium : 0.091					
		Medium : 0.052				Grafting technique × medium : 0.074					

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 16a: Effect of scion length, grafting technique and media composition on per cent aseptic culture using forced (*in vivo*) scion source (S_2)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		67.21	64.43	64.20	62.21	64.51	65.55	64.21	64.14	62.32	64.06
L_2		63.15	63.09	62.12	60.17	62.13	65.54	65.07	62.32	61.94	63.72
L_3		61.21	59.21	58.85	58.24	59.38	62.67	62.22	60.10	59.79	61.20
Mean		63.86	62.24	61.72	60.21		64.59	63.83	62.19	61.35	
C.D($p \leq 0.05$)		Scion length : 0.409				Scion length × grafting technique : 0.578					
		Grafting technique : 0.334				Scion length × medium : 0.817					
		Medium : 0.472				Grafting technique × medium : NS					

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 16b: Effect of scion length, grafting technique and media composition on per cent graft success using forced scion source (S_2)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		16.25	17.77	17.32	16.63	16.99	12.49	14.82	13.71	13.05	13.52
L_2		17.27	18.55	15.32	14.35	16.37	12.00	13.60	12.83	12.66	12.77
L_3		11.25	12.03	11.49	10.93	11.43	9.63	11.06	10.85	8.04	9.90
Mean		14.92	16.12	14.71	13.97		11.37	13.16	12.46	11.25	
C.D ($p \leq 0.05$)		Scion length : 0.657				Scion length × grafting technique : 0.929					
		Grafting technique : 0.536				Scion length × medium : N.S					
		Medium : 0.758				Grafting technique × medium : N.S					

Table 16c: Effect of scion length, grafting technique and media composition on per cent necrosis using forced scion source (S_2)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		49.74	20.03	25.79	50.40	36.49	56.64	16.18	29.18	7.15	27.29
L_2		40.24	20.69	50.36	0.00	27.82	52.12	12.02	43.21	31.94	34.82
L_3		65.98	0.00	66.31	0.00	33.07	61.22	15.09	53.22	39.14	42.17
Mean		51.99	13.57	47.49	16.80		56.66	14.43	41.87	26.08	
C.D ($p \leq 0.05$)		Scion length : 1.310				Scion length × grafting technique : 1.853					
		Grafting technique : 1.070				Scion length × medium : 2.621					
		Medium : 1.513				Grafting technique × medium : 2.140					

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 16d: Effect of scion length, grafting technique and media composition on vitrification (%) using forced scion source (S_2)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		24.17	31.58	18.56	27.37	25.42	34.33	39.45	26.09	34.42	33.57
L_2		19.51	26.35	15.19	22.85	20.98	27.15	36.81	19.11	31.66	28.68
L_3		25.18	37.05	17.76	30.33	27.58	31.97	33.25	26.22	30.46	30.48
Mean		22.95	31.66	17.17	26.85		31.15	36.50	23.81	32.18	
C.D ($p \leq 0.05$)		Scion length : 1.099				Scion length × grafting technique : 1.555					
		Grafting technique : 0.898				Scion length × medium : 2.199					
		Medium : 1.269				Grafting technique × medium : 1.795					

Table 16e: Effect of scion length, grafting technique and media composition on vigour (3 point scale) using forced scion source (S_2)

Scion length	Grafting technique	G_1				Mean	G_2				Mean
	Medium	M_1	M_2	M_3	M_4		M_1	M_2	M_3	M_4	
L_1		1.95	2.33	1.86	2.10	2.06	1.41	1.71	1.57	1.50	1.55
L_2		2.09	2.34	2.42	2.50	2.34	1.43	1.95	1.30	2.13	1.70
L_3		1.68	1.93	1.53	2.15	1.82	1.03	1.00	1.56	2.14	1.43
Mean		1.91	2.20	1.94	2.25		1.29	1.55	1.48	1.92	
C.D ($p \leq 0.05$)		Scion length : 0.054				Scion length × grafting technique : 0.077					
		Grafting technique : 0.044				Scion length × medium : 0.109					
		Medium : 0.063				Grafting technique × medium : 0.089					

L_1 = <5mm	L_2 =5-10mm	L_3 = 10-15mm	
G_1 = Wedge grafting	G_2 = Horizontal grafting		
M_1 = MS medium + 20 g/l sucrose	M_2 = MS liquid medium + 20 g/l sucrose	M_3 = MS medium + 40 g/l sucrose	M_4 = MS liquid medium + 40 g/l sucrose

Table 17a: Effect of pre-treatment of various growth regulators on ranked treatment combination of scion origin, scion length, grafting technique and media composition on per cent aseptic cultures and graft success of pear cv. Patharnakh grafted on Kainth rootstock

Treatment No.	Pre-treatment	Aseptic cultures (%)		Graft success (%)	
		$S_1G_1L_2M_2$	$S_2G_2L_3M_4$	$S_1G_1L_2M_2$	$S_2G_2L_3M_4$
T_1	**TDZ (0.25 mg/l)**	85.23	62.48	36.20	10.16
T_2	**TDZ (0.5 mg/l)**	87.41	65.32	38.41	13.06
T_3	**TDZ (0.75 mg/l)**	87.46	65.61	37.39	11.78
T_4	**2,4-D (5.0 mg/l)**	75.47	54.92	34.83	9.63
T_5	**2,4-D (7.5 mg/l)**	75.41	53.26	36.91	11.57
T_6	**2,4-D (10.0 mg/l)**	74.43	51.26	35.55	11.46
T_7	**TDZ (0.25 mg/l) + 2,4-D (5.0 mg/l)**	82.56	60.13	37.65	14.76
T_8	**TDZ (0.25 mg/l) + 2,4-D (7.5 mg/l)**	79.67	57.18	40.69	16.36
T_9	**TDZ (0.25 mg/l) + 2,4-D (10.0 mg/l)**	75.91	55.07	38.19	16.24
T_{10}	**TDZ (0.5 mg/l) + 2,4-D (5.0 mg/l)**	88.48	58.59	41.00	17.28
T_{11}	**TDZ (0.5 mg/l) + 2,4-D (7.5 mg/l)**	86.43	60.46	42.34	19.87
T_{12}	**TDZ (0.5 mg/l) + 2,4-D (10.0 mg/l)**	80.51	58.61	39.97	18.32
T_{13}	**TDZ (0.75 mg/l) + 2,4-D (5.0 mg/l)**	88.78	65.41	40.50	18.86
T_{14}	**TDZ (0.75 mg/l) + 2,4-D (7.5 mg/l)**	86.52	60.98	39.16	18.45
T_{15}	**TDZ (0.75 mg/l) + 2,4-D (10.0 mg/l)**	81.03	60.34	39.03	17.87
T_{16}	**TDZ (0.0 mg/l) + 2,4-D (0.0 mg/l)**	78.68	59.79	33.71	8.04
	Mean	**82.12**	**59.34**	**38.22**	**14.61**
	C.D (p≤0.05)	**2.616**	**2.759**	**2.312**	**2.307**

$S_1G_1L_2M_2$ *In vitro* scions of 5-10 mm length wedge grafted on Kainth and allowed to grow in MS liquid containing 20 g/l sucrose

$S_2G_2L_3M_4$ *In vivo* scions of 10-15 mm length horizontal grafted on Kainth and allowed to grow in MS liquid medium containing 40 g/l sucrose

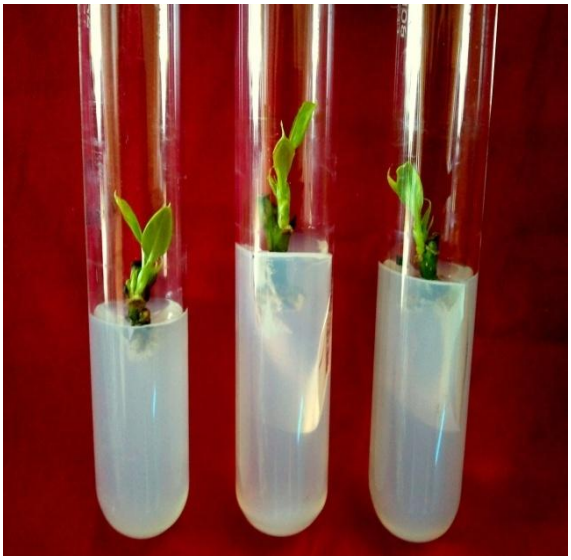
Table 17b: Effect of pre-treatment of various growth regulators on ranked treatment combination of scion origin, scion length, grafting technique and media composition on per cent necrosis and vitrification of pear cv. Patharnakh grafted on Kainth rootstock

Treatment No.	Pre-treatment	Necrosis (%)		Vitrification (%)	
		$S_1G_1L_2M_2$	$S_2G_2L_3M_4$	$S_1G_1L_2M_2$	$S_2G_2L_3M_4$
T_1	**TDZ (0.25 mg/l)**	4.59	25.31	7.10	47.96
T_2	**TDZ (0.5 mg/l)**	3.75	21.22	6.16	42.18
T_3	**TDZ (0.75 mg/l)**	1.65	15.45	6.12	42.02
T_4	**2,4-D (5.0 mg/l)**	6.26	35.45	16.09	47.16
T_5	**2,4-D (7.5 mg/l)**	7.43	32.38	12.93	52.51
T_6	**2,4-D (10.0 mg/l)**	8.57	35.69	13.33	56.18
T_7	**TDZ (0.25 mg/l) + 2,4-D (5.0 mg/l)**	5.12	29.42	12.11	47.34
T_8	**TDZ (0.25 mg/l) + 2,4-D (7.5 mg/l)**	6.19	32.54	9.04	43.27
T_9	**TDZ (0.25 mg/l) + 2,4-D (10.0 mg/l)**	6.93	38.65	8.97	49.57
T_{10}	**TDZ (0.5 mg/l) + 2,4-D (5.0 mg/l)**	5.15	27.54	8.10	35.18
T_{11}	**TDZ (0.5 mg/l) + 2,4-D (7.5 mg/l)**	6.15	29.32	5.48	33.02
T_{12}	**TDZ (0.5 mg/l) + 2,4-D (10.0 mg/l)**	7.26	31.86	6.16	39.40
T_{13}	**TDZ (0.75 mg/l) + 2,4-D (5.0 mg/l)**	4.62	22.41	7.81	28.86
T_{14}	**TDZ (0.75 mg/l) + 2,4-D (7.5 mg/l)**	6.64	27.31	4.11	34.48
T_{15}	**TDZ (0.75 mg/l) + 2,4-D (10.0 mg/l)**	8.08	36.34	4.56	42.61
T_{16}	**TDZ (0.0 mg/l) + 2,4-D (0.0 mg/l)**	6.71	39.14	15.94	54.46
	Mean	5.94	30.00	9.00	43.51
	C.D($p \leq 0.05$)	1.599	3.543	2.143	3.003

$S_1G_1L_2M_2$ *In vitro* scions of 5-10 mm length wedge grafted on Kainth and allowed to grow in MS liquid containing 20 g/l sucrose

$S_2G_2L_3M_4$ *In vivo* scions of 10-15 mm length horizontal grafted on Kainth and allowed to grow in MS liquid medium containing 40 g/l sucrose

A. Forced cutting of Patharnakh



B. Explant establishment on MS medium containing BAP (1.5mgl^{-1}) + IBA (0.01mgl^{-1})

C. Proliferation on WPM containing BAP (2.5mgl^{-1})

D. Rooting on ½ MS containing IBA (1.0mgl^{-1})

Plate I: *In vitro* propagation of Patharnakh (Forced) pear

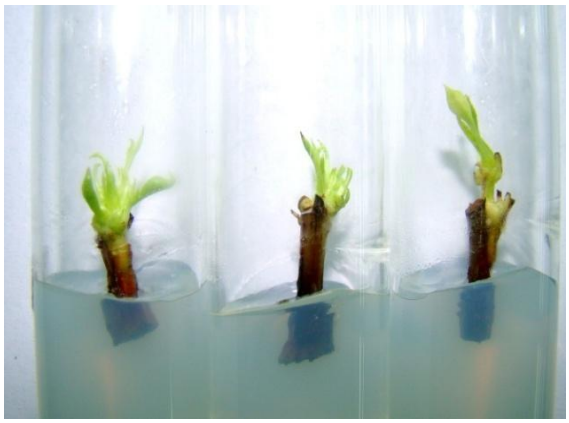
A. Establishment of Patharnakh using Murashige and Skoog medium fortified with BAP ($1.5mgl^{-1}$) and IBA ($0.01mgl^{-1}$)

B. Proliferation of Patharnakh using Woody Plant medium fortified with BAP ($5.0mgl^{-1}$)

C. Rooting of Patharnakh using Half strength Murashige and Skoog medium containing IBA ($1.0mgl^{-1}$)

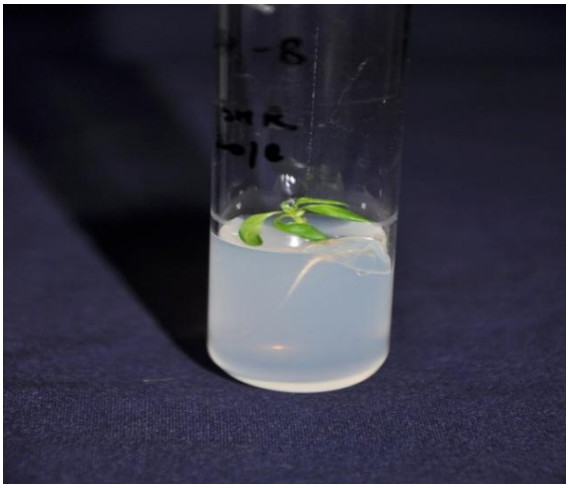
Plate II: *In vitro* propagation of Patharnakh (active) pear

A. Forced cutting of Kainth



B. Establishment on MS containing BAP ($1.5mgl^{-1}$) and IBA ($0.25mgl^{-1}$)

C. Proliferation on WPM fortified with BAP ($3.0mgl^{-1}$)

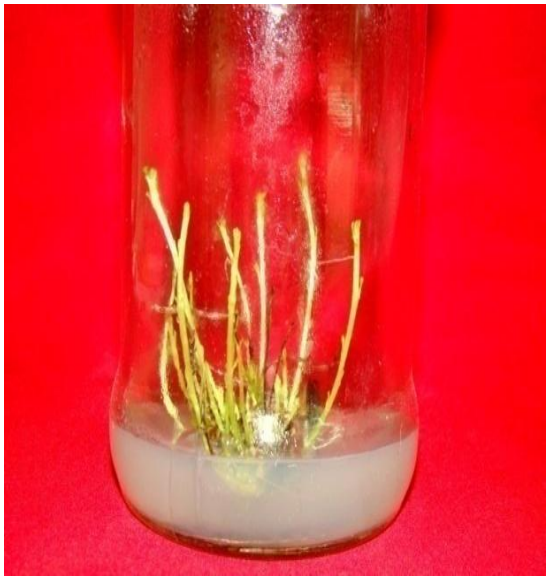


D. Rooting on ½ MS medium containing IBA ($0.1\ mgl^{-1}$)

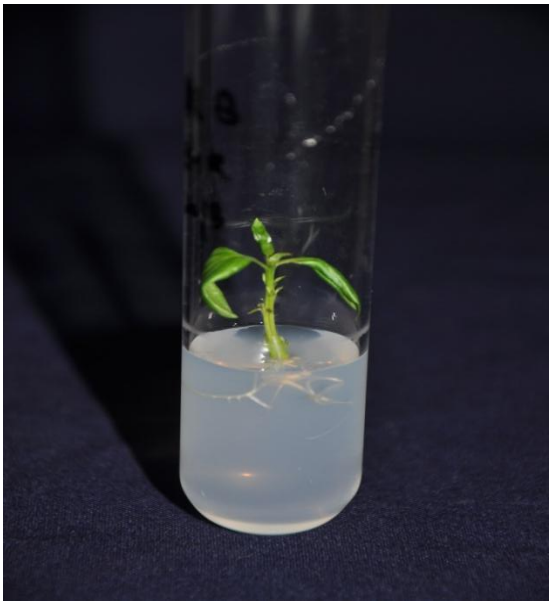
E. Rooting on ½ MS medium containing NAA ($1.0mgl^{-1}$)

Plate III: *In vitro* propagation of Kainth (forced) pear

A. Establishment on MS medium fortified with BAP (1.5mgl^{-1}) and IBA ((0.25mgl^{-1})



B. Proliferation of Kainth using WPM fortified with BAP (3.0mgl^{-1})

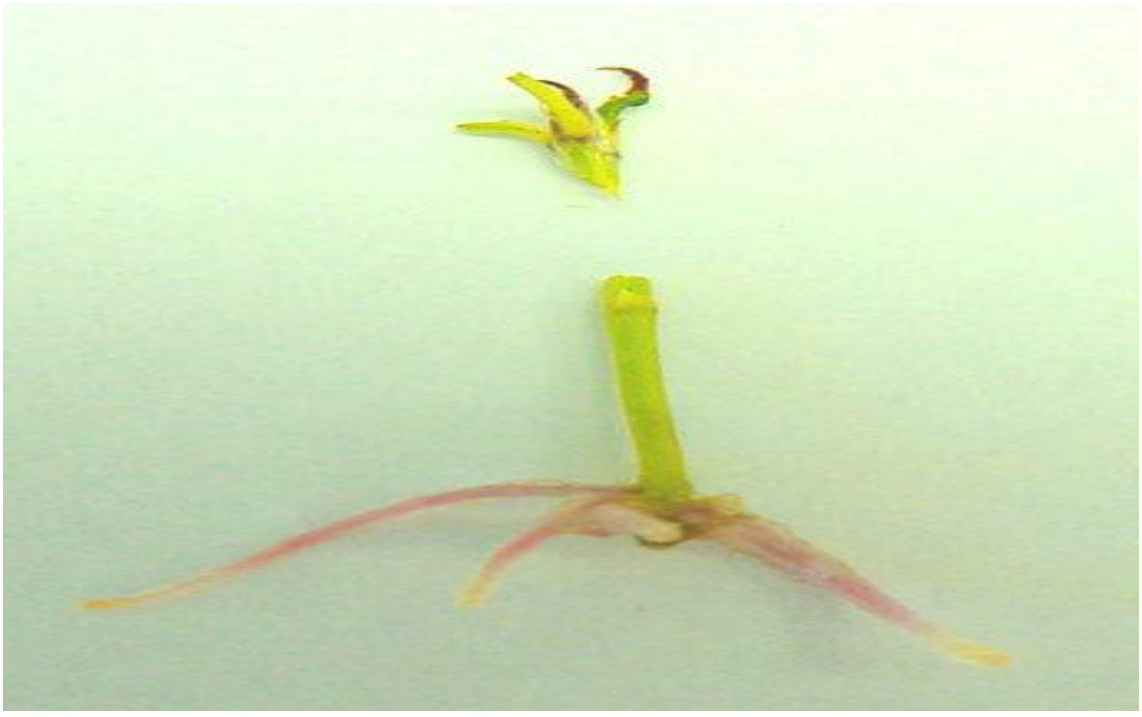


C. Rooting in Kainth in ½ MS containing IBA (0.1mgl^{-1})

D. Rooting in Kainth using ½ MS fortified with NAA (1.0mgl^{-1})

Plate IV: *In vitro* propagation of Kainth (active) pear

A. Wedge grafting



B. Horizontal grafting

Plate V: Types of shoot-tip grafting used

A. Successful micrograft

B. Necrotic grafted plantlet

C. Vitrified micrograft

D. Vigorous micrograft

Plate VI: Shoot tip grafting using *in vitro* scions

A. Successful micrograft

B. Necrotic grafted plantlet

C. Vitrified plantlet

D. Vigorous micrograft

Plate VII: Shoot-tip grafting using *in vivo* (forced) scions

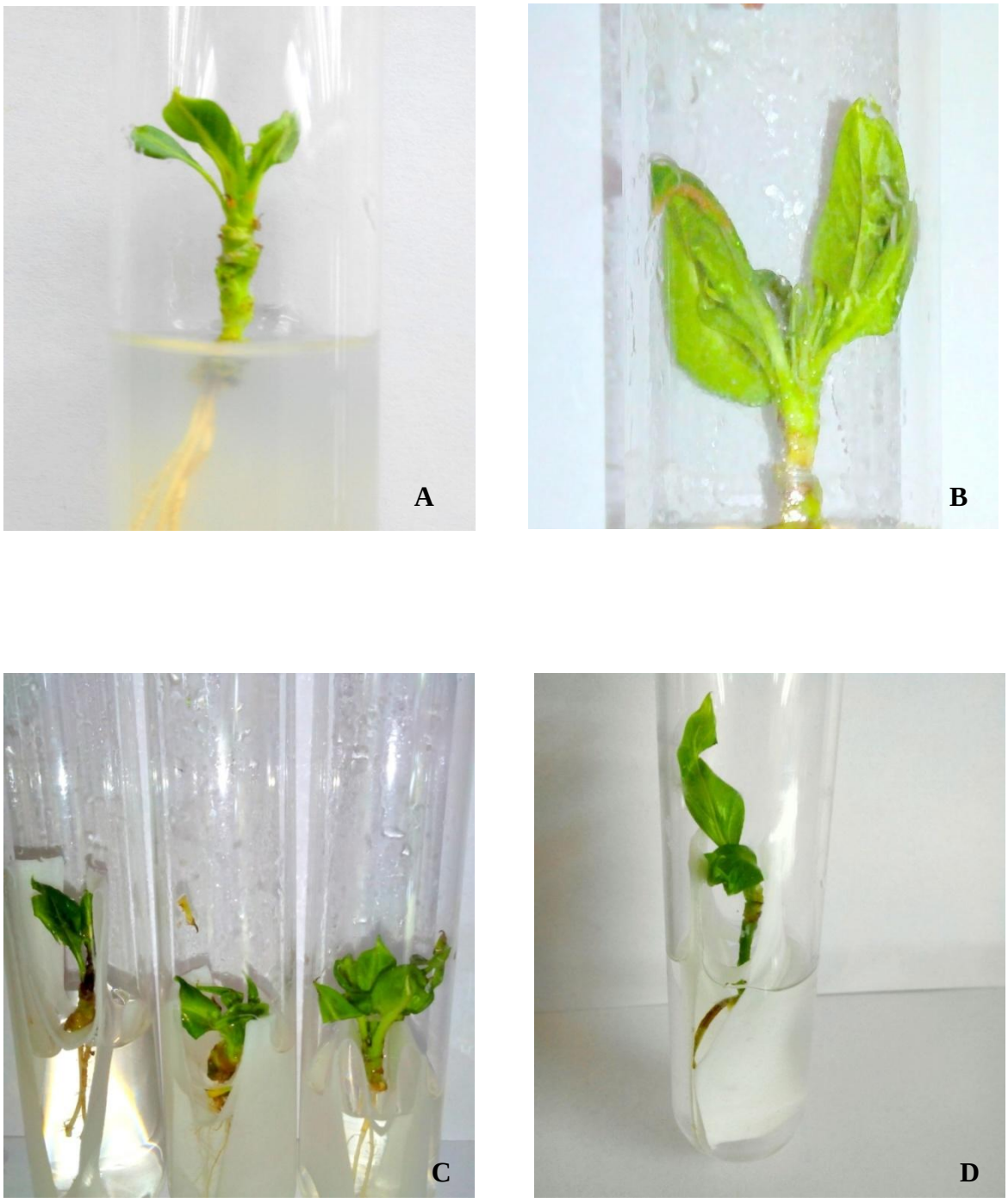


Plate 8: Successful micrografts after pre-treatment

VITA

Name of student	:	Haseeb ur Rehman
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University and	**:**	SKUAST-Shalimar-Srinagar
Year of award	**:**	2010
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Title of Master's thesis	**:**	Standardization of leaf sampling technique in plum and apricot
Ph.D	**:**	Horticulture (Fruit Science)
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Year of award	**:**	2014
OCPA	**:**	8.12
Title of Ph.D thesis	**:**	*In vitro* propagation and shoot-tip grafting of Patharnakh (*Pyrus pyrifolia* (Burm F.) Nakia) pear

Awards/ Distinction/ Fellowships/ Scholarships

	:	ICAR non-JRF
	:	ICAR non-SRF
	:	ASRB NET (2 times)
	:	Certificate of merit for highest OGPA in M.Sc.
	:	Moulana Azad National Fellowship holder