

MICROPROPAGATION OF PROSOPIS CINERARIA (L) DRUCE

BY

SARITA SHARMA

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IN

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CCS Haryana Agricultural University**

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TO

MY

PARENTS

CERTIFICATE-I

This is to certify that this thesis entitled, "Micropropagation of Prosopis cineraria (L) Druce", submitted for the degree of Master of Science in the subject of Genetics of CCS Haryana Agricultural University, Hisar, is a bonafide research work carried out by Miss Sarita Sharma 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.


(R.C. YADAV)
Major Advisor

CERTIFICATE-II

This is to certify that this thesis entitled, "Micropropagation of Prosopis cineraria (L) Druce", submitted by Miss Sarita Sharma to the CCS Haryana Agricultural University, Hisar, in partial fulfilment of the requirements for the degree of Master of Science, in the subject of Genetics has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

R. Yadav

MAJOR ADVISOR

[Signature] 13/10/92
EXTERNAL EXAMINER

J. K. Soree

HEAD OF THE DEPARTMENT

[Signature] 28/11

DEAN, POST-GRADUATE STUDIES

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(SARITA SHARMA)

ABBREVIATIONS

BA	6-Benzylaminopurine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	α -naphthalene acetic acid
NOA	Naphthoxy acetic acid
PVP	Polyvinylpyrrolidone
WM	White (1934) medium
ZEA	Zeatin 6-[4-hydroxy-3-methyl-but-2-enyl]aminopurine
2,4-D	(2,4-Dichlorophenoxy)acetic acid

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INTRODUCTION

Propagation of trees has always fascinated man for a long time. In the face of growing world population the demand for wood and forest products is predicted to increase greatly over the next few decades and shortages have been forecasted by the end of this century (Keays, 1974). Therefore, concerted efforts must be made to evolve methods for mass propagation and production of short duration tree suitable for agroforestry, fuel, timber and fodder. About 15% of the world's annual fuel comes from biomass and wood is the largest biomass source of fuel energy (Anon, 1980).

For centuries, conventional techniques have contributed much to the improvement of tree species. However, the resources of useful genetic variation are nearing exhaustion. Selection efficiencies are low, and the breeding cycle is very long. Therefore, it becomes imperative to combine traditional breeding methods with new biotechnological techniques for tree improvement. Karnosky (1981) suggested tissue culture as a method for forest tree improvement.

This technique has now proved to be an efficient tool for rapid clonal propagation of several important plant species. However, tissue culture of forest trees has lagged behind that of many agricultural crops-probably due to the recalcitrant nature of woody plants.

Prosopis cineraria (L.) Druce, a leguminous tree, selected for the present investigation is a multipurpose vegetation element in arid and semi-arid countries. It provides man with firewood, timber, food, animal fodder, gums and tannins (Leakey and Last, 1980). It is a nitrogen fixer and is considered the best choice for social and agroforestry plantations in arid and semi-arid regions. Natural reproduction of this tree species is mainly accomplished by coppicing and root suckers (Prakash and Hocking, 1986). Under natural environment, germination occurs only under exceptionally favourable moisture conditions e.g. moist depressions and places close to streams and rivers. Vegetative propagation with cuttings is difficult as rooting is poor. This tree is slow growing and suffers from several diseases and pests. So germplasm with superior traits like fast growth and resistance to salinity, high temperature, water stress, disease and pests have to be identified and multiplied. Plant tissue culture technology has the viable potential in producing thousands of plants which are true to type and, therefore, offers an alternative for multiplying elite germplasm. Keeping this in view, the present study was planned with the following objectives:

- 1 To develop protocols for callus initiation and plant regeneration in Prosopis cineraria .
- 2 To transfer regenerated plants to soil, if possible.

REVIEW OF LITERATURE

Tissue culture techniques are becoming useful tools for rapid clonal multiplication of plants. The commercial feasibility of this technique has been demonstrated in many diverse genera including a few forest trees. The advantage of tissue culture cloning over conventional propagation technique is to reduce the time needed for achieving large scale propagation of plants which are identical to their parents.

Development of buds and apices into shoots and rooting of the shoots has been achieved for many herbaceous species and also for some angiosperms. Gautheret (1934, 1940) was the first to obtain calli from cambial explants of angiosperm and conifer trees. Fully organised plantlets of tree species propagated in vitro and capable of subsequent transfer to soil were first obtained in Populus tremuloides (Winton, 1968). In gymnosperms first true plantlet was obtained from Pinus sylvestris (Winton, 1978). There was a gap of 30-40 years between initial attempts

and the achievement of propagation of plantlets suitable for cultivation. This gap may be due to relatively slow response of woody species to culture conditions than herbaceous plants.

The genetically most conservative method for in vitro propagation is probably culturing of apical/axillary buds and shoot tips.

A large number of angiosperm species have been subjected to in vitro culture studies. However, the review that follows mainly relates to woody angiosperm tree species.

2.1 Factors influencing plant regeneration

The in vitro growth and development of a plant is determined by a number of complex factors:

- i) Genotype of the donor plant
- ii) Culture medium
- iii) Age of the cultures
- iv) Choice of explant
- v) Light and temperature conditions etc.

2.1.1 Genotype

Coleman and Ernst (1989) observed genotypic differences in shoot regeneration for 16 genotypes of Populus deltoides when cultured on medium supplemented with 0.5 mg/l Zeatin and found that six genotypes were highly recalcitrant and failed to regenerate shoots. However, percentage regeneration from explants in four genotypes was more than 50%. Han et al. (1990) observed differential response in shoot explants regenerated from two clones of Robinia pseudoacacia trees.

One clone consistently out-performed the other with respect to shoot proliferation and proportion of regenerated shoots producing roots. Coleman and Ernst (1990) also reported significant differences between 4 genotypes of Populus deltoides in the production and growth of axillary shoots. Chung and Chung (1991) observed that average number of shoots per nodule from Populus euramericana clone ECo 28 and P. nigra x P. maximowiczii clone 62-9 were 24 and 27 respectively. In a comparative study of 30 yr. old Larix decidua, L. leptolepis, L. eurolepis and L. laricina; Bonga and Pond (1991) found that explants from L. eurolepis and L. decidua produced a high number of cultures with adventitious buds that elongated into shoots; those from L. leptolepis were less productive and those from L. laricina failed to form adventitious buds. Under optimal conditions, adventitious shoot regeneration from in vitro leaves of several pear cultivars (Pyrus communis L.) 'Comice', 'Passe - crassane', 'Williams' and 'Conference', ranged from 60% to 97%, with the mean number of shoots per regenerating leaf ranging from 3.2 to 6.6 (Leblay et al., 1991).

McComb and Bennett (1982) reported that lines from adult Eucalyptus marginata trees in culture gave 0-90% rooting. Similarly, growth in culture and rooting rates were both markedly affected by the source genotype in case of explants obtained from Quercus petraea. Clones 85 and 17.04.6 which had rooting rates of 100% and 58.3% respectively (San-Jose et al., 1990).

2.1.2 Culture Media

2.1.2.1 Nutrients

The media used most frequently and with greatest success are Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), woody plant medium (WPM) (Lloyd and McCown, 1980), and broad leaved tree medium (BTM) (Chalupa, 1981,

1983, 1984). Gresshoff and Doy (GD) medium (Gresshoff and Doy, 1972), Wolter and Skoog (WS) medium (Wolter and Skoog, 1966) and white medium have been used less frequently.

In Populus alba x Populus glandulosa callus was initiated from internodal tissue on modified MS medium containing Kinetin and 2,4-D while for shoot regeneration woody plant medium (WPM) supplemented with NAA and BA was required (Kim et al., 1988). Of various basal media used for culturing axillary buds of oak (Quercus robur), GD medium gave the best result. For leaf explants Dustan and Short's (DS) medium supplemented with NAA and BA produced shoot buds (Civinova and Sladky, 1987). Gamborg's B₅ medium (Gamborg et al., 1968) was used by Mukhopadhyay and Mohan Ram (1981) for culturing Dalbergia sissoo. Whereas Datta and Datta (1983) obtained multiple shoots in D. sissoo on modified MS medium (MS medium + vitamins of Gamborg B₅ medium) containing NAA.

Chalupa (1983) reported high multiplication rate of Betula pendula and B. pubescens on BTM and WPM containing a low level of cytokinin while in Fraxinus excelsior shoot formation from axillary buds was best on WPM and on modified MS medium supplemented with cytokinin. In Liquidambar styraciflua prolific shoot production occurred with WPM supplemented with BA (Brand and Lineberger, 1988). Jang et al. (1988) found that in Populus glandulosa, cell proliferation and callus formation were satisfactory on MS medium with Zeatin and NAA and WPM with Zeatin and NAA/IAA. Shoot induction from callus was optimum on MS medium with Zeatin and NAA/IAA and WPM with Zeatin and NAA.

BTM and GD media supplemented with BA (0.55 mg/l) were used by Johnson and Walker (1990) for culturing Quercus lobata stem segments. They observed that greater proportion of segments on BTM had developed buds, but more

segments on GD showed shoot growth. In Robinia pseudoacacia MS medium has been shown to support shoots under BA and NAA treatment (Hlan et al., 1990). Regenerated shoots produced roots on 0.1 strength MS medium containing IBA. Driver and Kuniyuki walnut medium was used by Coleman and Ernst (1990) for culturing Populus deltoides. In Eucalyptus tereticornis callus was initiated from leaf and stem explants on B₅ medium while for shoot initiation modified woody plant medium (MWP) was required (Subbaiah and Minocha, 1990). Mathur and Mukunthakumar (1992) reported axillary shoot proliferation from nodal explants excised from Bauhinia variegata and Parkinsonia aculeata on MS medium supplemented with BA.

Duhoux and Davies (1985) reported rhizogenesis of shoot buds of Acacia albida with increase in concentration of sucrose (50 g/l) in LS (Linsmaier and Skoog, 1965) medium. Tomar and Gupta (1988) found that in Albizzia richardiana, sucrose at the 2% level in MS medium was optimal for embryogenesis and 4% for shoot bud formation. High concentration of sucrose (6-10%) caused browning of callus and inhibited the differentiation of embryos.

Ammonium and total nitrogen play an important role in growth and differentiation. Anjani Kumar (1989) reported in Dalbergia sissoo that calli grown in the absence of AgNO₃ turned tan in colour and showed signs of senescence after 45 days growth, while those grown in the presence of AgNO₃ remained yellowish white. The effect of medium nitrogen sources on the recalcitrant nature of Prosopis alba clone B2 V50 in tissue culture were compared involving shoot development using axillary bud explants (Green et al., 1990). A comparison between amide and ureide nitrogen sources showed that allantoin, a ureide was an acceptable replacement for asparagine or glutamine. Allantoin,

asparagine and glutamine could be used as the sole nitrogen sources. When allantoin at a concentration of 20 mM was used, although shoots were consistently induced, all explants showed complete shoot-tip necrosis after 12 weeks of in vitro culture. Intermediate NH_4^+ concentrations were suitable for adventitious shoot regeneration from in vitro leaves of several pear cultivars. Optimal regeneration occurred on media with a 1:3 $\text{NH}_4^+/\text{NO}_3^-$ ratio (Leblay et al., 1991). Nuntila et al. (1991) reported that in Betula pendula callus, maximum embryo production can be achieved with 35 mM total inorganic nitrogen and 20.8 g/l sucrose in the medium.

Arya et al. (1978) reported that in Aegle marmelos incorporation of L-tyrosine increased the number of shoots per explant. Lakshmi Sita and Shobha Rani (1985) obtained multiple shoots in Eucalyptus grandis when thiamine was used. In Eucalyptus camaldulensis, Kumar and Raman (1989) observed that cotyledonary nodes cultured on MS medium containing 5 mg/l BA, 160 mg/l adenine sulphate and 170 mg/l sodium dihydrogen orthophosphate developed shoot buds from callus. However the inclusion of riboflavin affects the form of the roots in Eucalyptus ficifolia. Shoots rooted without riboflavin produced many fibrous roots near the surface of the agar. In the presence of riboflavin, one to two strong, thick roots grew from the base of the shoots (De Fossard, 1978, Gorst et al., 1981, 1983).

2.1.2.2 Growth regulators

Durzan and Lopushanski (1975) established American elm callus from hypocotyl segments placed on agar medium enriched with Kinetin and NAA. Chalupa (1975) reported rapid growth of callus in twigs of Ulmus campestris with addition of 1-5 mg/l NAA, BA and 2,4-D to LS medium. Bhargava et al.

(1981) observed callus formation in hypocotyl explant of Dalbergia sissoo on MS medium with kinetin (0.2 mg/l) and 2,4-D (8.0 mg/l). Rao et al. (1988) found that in Azadirachta indica combination of 2,4-D and IAA was the most effective in producing callus. Devi and Nataraja (1987) reported slight callusing in Dalbergia latifolia in almost all the cultures and roots regenerated directly from the explants on MS medium with IAA alone and cultures with 2,4-D or kinetin produced only callus. King and Morehart (1987) found that callus growth was positively affected by the presence of both an auxin and cytokinin in the medium in Acer rubrum. Optimal growth depended on the ratio of cytokinin/auxin as well as the total amount of plant growth regulators in the medium.

Rao and Bapat (1978) demonstrated the regeneration of shoot buds in hypocotyledonary segments on media supplemented with IAA, IBA, NAA, NOA (1 mg/l each). In Dalbergia sissoo, supplementation of the medium with NAA alone or in combination with BA resulted in shoot production (Mukhopadhyay and Mohan Ram, 1981). Rao et al. (1984) reported that in Dalbergia latifolia, decrease in NAA concentration ensured shoot formation. Kackar et al. (1991) found that single node segments of Prosopis cineraria cultured on MS basal medium containing 3 mg/l each of NAA and NOA produced an axillary shoot. They also observed development of adventitious buds in root segments of Prosopis cineraria on MS medium containing NOA (Kackar et al., 1992).

In Aegle marmelos callus tissue, shoot formation was induced with high Kinetin concentration in MS medium devoid of auxin or with its low concentration (Arya et al., 1978). Added concentration of Kinetin in the medium increased the number of shoots from stem and hypocotyl segments of Albizia lebbek, but reduced the shoot length and incorporation of IBA in low concentrations enhanced shoot length

(Arya et al., 1978). In Betula pendula cell culture, embryoids were formed in liquid and on solidified N_7 medium containing Kinetin (0.5 mg/l) and 2,4-D (2 mg/l) while the embryoids germinated on hormone free medium (Kurten et al., 1990). Bhargava et al. (1981) observed direct differentiation of shoots from hypocotyl segments of Albizzia lebbek seedlings on MS medium with Kinetin or a combination of Kinetin and IAA. Goyal and Arya (1981) reported differentiation of shoots and roots from hypocotyl of Prosopis cineraria on MS medium with different concentrations and combinations of Kinetin, NAA and IAA. Indole acetic acid (3.0 mg/l) with Kinetin (0.05 mg/l) was found to be the best suited for multiple shoot differentiation in bud explants of P. cineraria (Goyal and Arya, 1984). Benzyladenine has been found to be the most effective cytokinin for shoot formation in many tree species such as Dalbergia latifolia (Devi and Nataraja, 1987), Betula celtiberica (Perez and Postigo, 1989), Eucalyptus globulus (Trindade et al., 1990), Melia azedarach (Ahmed et al., 1990) and Tamarindus indica (Jaiwal and Gulati, 1991). Joshi et al. (1991) observed induction of multiple shoots from cotyledonary and epicotyledonary nodes of Anogeissus pendula on MS medium containing BA (1.0 mg/l) and IAA (0.1 mg/l). Ramesh and Padhya (1990) reported development of adventitious shoot buds in Azadirachta indica cultures on a medium supplemented with Kinetin and BA.

Bhargava et al. (1981) observed rooting in Albizzia lebbek on MS medium with Kinetin and IAA while half MS medium supplement with IBA along with Kinetin was used for rooting in Anogeissus pendula (Joshi et al., 1991).

MS media supplemented with IBA alone has been successfully used in Melia azedarach (Ahmed et al., 1990). Betula celtiberica (Perez and Postigo, 1989) for rooting regenerated shoots. In Tamarindus indica, MS medium supplemented with IAA alone have been used for rooting of shoots (Jaiwal and Gulati, 1991)

but in Melia azedarch this media was found to be ineffective in inducing roots (Ahmed et al., 1990).

In case of Prosopis cineraria use of auxins like IAA and IBA (0-2 mg/l) single or in combination has been found to result in no or poor root initiation (Kackar et al., 1991). Adventitious root formation in regenerated shoots of Prosopis cineraria has been achieved in white's medium containing IBA and Kinetin as reported by Goyal and Arya (1981, 1984) and Arya and Shekhawat (1986) whereas Kackar et al. (1991, 1992) used MS medium supplemented with NOA (3.0 mg/l) for rooting in regenerated shoots of P. cineraria.

2.1.2.3 Other additives

Rangaswamy and Rao (1963) reported callus initiation and establishment from endosperm tissue of sandalwood on modified White's medium (White, 1934) supplemented with yeast extract, 2,4-D and Kinetin. In Dalbergia lanceolaria addition of yeast extract to MS medium supplemented with NAA and Kinetin increased the number of differentiated roots (Anand and Bir, 1984). In Eucalyptus camaldulensis, Kumar and Raman (1989) obtained callus from embryo tissue cultured on MS medium supplemented with casein hydrolysate (1 g/l) and 2,4-D (2 mg/l). Simola (1985) obtained plantlets in Betula pendula on N₇ medium supplemented with casein hydrolysate, Zeatin or Zeatin riboside. In Dalbergia sissoo. Bhandary (1988) obtained shoots from excised cotyledons cultured on MS medium with casein hydrolysate and BA. In Prunus amygdalus Mehra and Mehra (1974) reported that casein hydrolysate and coconut milk were essential for callusing and differentiation. In Eucalyptus, Aneja and Atal (1969) and Kitahara and Caldas (1975) found that callus growth required coconut milk, however other workers have not used coconut milk for callus growth (Lakshmi Sita, 1979, Ouyang et al., 1980, 1981, Bennett and McComb, 1982).

Different workers have used coconut milk at varying concentrations for callus formation and shoot regeneration. Greenish white, soft and subculturable callus was formed in Dalbergia latifolia on addition of coconut water (15%) (Devi and Nataraja, 1987) and multiple shoot development was reported from axillary buds of Acacia auriculiformis on addition of coconut milk (5 or 10%) (Mittal et al., 1989).

In woody species browning of callus and medium is a common problem. Addition of soluble PVP (Rai and Chandra, 1988) or addition of activated charcoal (Sharma et al., 1988) has been found to overcome this problem. Sometimes quick transfer of calli to fresh medium is reported to minimize browning (Rai and Chandra, 1988).

Activated charcoal has also been reported to induce rooting in Prosopis cineraria (Kackar et al., 1991) and in red horse chestnut (Masubuchi, 1991).

2.1.3 Age of culture

Increased rooting frequency with increased time in culture is reported for Eucalyptus rudis, E. citriodora and E. marginata. Cultured shoots from a 20 year old E. citriodora did not root until the fourth subculture by which time they had been in culture for 240 days (Gupta et al., 1981), for E. rudis four month of subculture was necessary (Badia Nkanda, 1982). Similar results are shown for E. marginata where an improvement in rooting is seen after 12 months or more in culture. Paton et al. (1981) reported that this change in cultured material is presumably analogous to the rejuvenation obtained by grafting mature scions onto seedling stocks.

An improvement in rooting of shoot cultures with time has also been noted with other woody species. In Prunus padus, Chalupa (1983) obtained multiple shoots after the third transfer.

2.1.4 Choice of explant

Regeneration of explants depends on a number of factors. These involve the selection of organs that serve as tissue source, the season in which the explant is being obtained, the size of the explant, original position of the explant in the plant, age of the plant and the overall quality of the plant from which explants are to be obtained (Murashige, 1974).

2.1.4.1 Size of explants

Tomar and Gupta (1988) found that larger pieces of hypocotyl from Albizzia richardiana developed shoots with green calli. Okazova et al. (1967) also reported that smaller explants are more likely to form callus while the larger explants maintain greater morphogenetic potentiality. In Populus wilsoni, the time required to obtain culture decreased as the size of the explant increased (Rutledge and Douglas, 1988). Similar relationship between explant size and morphogenetic potentiality has been reported in the past (Vine, 1968; Smith and Murashige, 1982). Larger explants are sometimes easier to regenerate than smaller ones, perhaps due to the presence of more food reserves (Anderson, 1980).

2.1.4.2 Source of explant

Considerable difference in organogenetic capability between different parts of the plants have been noted. Such differences occur between neighbouring tissues or cells (Tran Thanh Van, 1980; Bilkey and Cocking, 1981), even within such a small organ as shoot apical meristem (Ball, 1980).

Volkaert et al. (1990) found that shoot development was slower for apical shoot explants than for nodal shoot explants of young oak (Quercus robur) seedlings. Rao et al. (1988) observed that cotyledons were the best source followed by leaves for callus induction and growth in Azadirachta indica explants.

Moon et al. (1987) concluded that axillary buds from base of the stem of Quercus acutissima showed multiple shoots production followed by those from the middle while rooting was best in buds taken from top of the stem. Similarly cultures originating from apices had a significantly higher multiplication rate than those originating from axillary buds excised from Fagus sylvatica while in Betula jackmontii, the explants derived from the base of shoots had a significantly higher multiplication rate as compared to top and central nodes (Vanderschaeghe and Debergh, 1989). Cresswell and Nitsch (1975) found that organ culture of nodes of Eucalyptus grandis permitted the rooting of only those nodes which were much beyond the cotyledonary node.

The capacity for clonal propagation is closely linked to the genetic and physiological factors that control the transition from juvenile to mature growth in the trees (Bonga, 1982). In general, the regenerative competence of juvenile material is much higher than that of mature explants.

Anand and Bir (1984) reported that callus developed from leaf and stem segments taken from mature trees of Dalbergia lanceolaria failed to differentiate while organogenesis occurred in calli developed from seedling segments. Arce and Balboa (1991) observed 80% regeneration rate of complete plant in juvenile material of Prosopis chilensis and 60% in 40 year old plant. Rehman et al. (1981) have found that nodal segments from mature tree of Hevea brasiliensis failed to produce plantlets while 6 to 8 week old seedling explants regenerated plantlets.

Similarly, explants from mature tree of Eucalyptus citridora required pre-treatment for induction of shoot buds but explants from seedling did not require any treatment (Gupta et al., 1981). San-Jose et al. (1990) observed no great

influence of the explant source on shoot multiplication rate in Quercus petraea and Volkaert et al. (1990) reported that seedling age had no significant effect on axillary shoot formation from apical and nodal shoot explants of Quercus robur seedling. Rao et al. (1985) reported that the percentage of rooting in adult derived shoots was less as compared to juvenile derived shoots in Eucalyptus grandis. However, in a few tree species some tissues remain capable of organogenesis for many years after the trees reach maturity. For example, shoot formation from terminal buds of 100 year old Tectona grandis (Gupta et al., 1980) and from leaf tissue of 60 year old Populus balsamifera (Bychenkova and David, 1978) has been reported.

2.1.4.3 The physiological state

The physiological state of the parent plant at the time of excising the explant has a strong effect on in vitro cell division and differentiation. Buds taken from plants (especially trees and Shrubs), which are still in a resting state (late autumn or early winter), are more difficult to culture in vitro than buds from plants which are no longer dormant (taken in spring, just before they are about to burst into growth) (Sussex and Clutter, 1959). Internodal explants taken from juvenile and adult branches of Robinia pseudoacacia and Castanea vulgaris exhibited seasonal variations in callus proliferation (Trippi, 1963). Bychenkova (1963) reported that growth of the callus was generally slow after bud break.

In Liquidambar styraciflua leaves from intact plants produced over four times more adventitious shoots than leaves from in vitro shoots and had a greater tendency to form shoots on the lamina. Leaves that were either 20% or 50% of full size and still actively expanding were superior to other developmental stages for shoot organogenesis (Brand and Lineberger, 1991).

2.1.5 Physical factors

It is extremely difficult to indicate whether a particular culture should be grown in the light or the dark, at high or low temperatures. It is usually best to choose those light and temperature conditions that are best for the growth and development of the individual experimental material in vivo.

For poplar cultures, 28°C was required for embryo induction, and 24-28°C for plantlet regeneration (Ba and Guo, 1983). Cornu and Chaix (1981) found that in Prunus avium, six month storage of cultures at 2°C without light doubled the proliferation rate of cultures after their return to normal temperature. Some clones survived more than one year in cold storage. They also observed that low light intensities and lower temperature (21°C) were most effective in promoting rooting of excised shoots. Whereas Druart et al. (1981) obtained high rooting percentages (90%), when the cultures stayed for 8-10 days in darkness at the beginning of the rooting stage and more roots were formed at 25°C. In Castanea sativa, Vieitez and Vieitez (1983) found that rooting percentage was low if the cultures were kept in darkness for 2 weeks prior to rooting.

2.2 Hardening of Regenerated Plants and Transfer to Soil

The ultimate success of any tissue culture programme depends upon the ability to transfer intact plants to soil.

Before transfer, the plants or the shoots should be rinsed to remove traces of agar, which might provide a substrate for microorganisms. Often rinsing is sufficient, but in some cases it is preferable to rinse the substrate with a suspension of fungicide (22.5 g/l Captan) before the planting (Bornman, 1983) or to spray the plants with a fungicide (0.01% wettable powder suspension of Benlate) once a week after planting out (Campbell and Durzan, 1976).

Plantlets or shoots produced in vitro must be acclimated gradually to withstand the less humid and higher light intensities existing outside the conditions in vitro. This can for example be accomplished by misting/spraying or by covering the pots. Different types of substrates have been used under the acclimatization period such as soil-vermiculite mixture (Goyal and Arya, 1981, 1983), sterilized sand (Bhansali et al., 1988) and soil (Kurten et al., 1990).

Durand-Cresswell et al. (1982) transferred in vitro raised eucalypts plantlets to "melfert balls" which are small unwoven bags containing pine bark, sphagnum peat, brown coal ash and osmocote fertilizer. For Eucalyptus marginata, McComb and Bennett (1982) used a mixture of two parts sand to one part sphagnum peat. Vieitez et al. (1986) obtained a survival rate of 34% when rooted chestnut plantlets were transferred to soil after 4 weeks in the root culture medium. An initial planting mixture of peat and perlite caused necrosis of the roots and base of the plantlets and better results were obtained with equal parts of soil and perlite. Once the plantlets were established the pots were transferred from the glasshouse to a shade house before planting out directly to soil. A method was developed to allow the Acacia koa plantlets to form normal well-branched roots with root hairs by growing in Hoagland's ^{solution} (Hoagland and Arnon, 1950) before transplanting to potting mix (Skolmen and Mapes, 1978). This method was later discarded when it was found that at least 50% of the plantlets survived if placed directly in potting mix and kept in polyethylene bags to reduce transpiration for 3-4 weeks while roots extended and new leaves formed (Skolmen, 1986).

Goyal and Arya (1983) transplanted Prosopis cineraria plantlets into small pots containing a sterile soil : Vermiculite mixture (3:1 v/v). Pots were covered with polyethylene bags to maintain high humidity. Plantlets were reared under

semi-controlled temperature (25-32°C) and light (3,000 lx) in a chamber. Upon the first sign of leaf proliferation plants were taken to a glass house. An economy in the cost of micropropagated plants could be achieved if the in vitro rooting stage could be omitted and shoots treated as tender cuttings and rooted in the glasshouse (Hutchinson, 1982). In Populus alba x P. grandidentata regenerated shoots were easily rooted in polyterra peat plugs in transparent plastic boxes. The rooted plantlets were subsequently transferred to pots containing an artificial potting mix (Son and Hall, 1990). For Quercus acutissima plantlets, a combination of vermiculite and 5 mg/l ancymidol provided the best growing medium and plantlets so propagated became acclimatized (from 78% humidity to a lower humidity) in about 9 days (Yamamoto et al., 1991).

MATERIALS AND METHODS

3.1 Material

3.1.1 Plant material

Prosopis cineraria (L.) Druce, a leguminous tree was selected for the present investigation. Various explants used for the callus initiation and plant regeneration are given in table 1.

Table 1 List of different explants and their sources in Prosopis cineraria (L.) Druce used in the present study

Sr.No.	Source of plant material	Explants
1.	Seedling (7-15 days old)	Cotyledon, hypocotyl, root and shoot tip.
2.	Juvenile tree (3 years old)	Axillary bud, young leaves, internodal segment and shoot tip of growing branches.
3.	Mature tree (9 years old)	Axillary bud, young leaves, internodal segment and shoot tip of growing branches.

Seeds and other plant material of Prosopis cineraria were provided by the Department of Forestry, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

3.1.2 Glasswares

All the glasswares used were of the borosilicate quality and obtained from either Borosil India or Corning Glass Company.

3.1.3 Chemicals

Throughout the investigation precaution was taken to use chemicals of high purity only. Fine chemicals such as growth hormones, vitamins, inositol, glycine etc. were obtained from Sigma Chemicals Company (USA), where as ordinary chemicals were purchased from BDH, Glaxo, E'Merck, Sisco Research Laboratories and Hi-Media.

3.2 Methods

3.2.1 Sterilization of glassware, culture media and explants

Glassware was thoroughly washed, rinsed with distilled water and dried in an oven for two hours at 140°C. Flasks and test tubes containing medium or distilled water were cotton plugged and steam sterilized in an autoclave at 1 kg/cm² pressure at 121°C temperature for 15 min. Petriplates, scalpels, forceps were heat sterilized at 180°C for 2-3 hours in an oven. Scalpels and forceps were also flame sterilized prior to use.

Explants were surface sterilized by a quick dip (for a few seconds) in 90% ethanol followed by 0.1% I₂ solution for 3-10 min, depending on the nature of the plant material and finally rinsed several times in sterilized distilled water.

In order to maintain aseptic conditions, all the manipulations were carried out in a laminar Air Work Station (Klenzaid).

3.2.2 Storage of culture media and stock solutions

The autoclaved media in tubes or conical flasks were stored in shade at 20-25°C in cold room and were used within a week of their preparation. Stock solutions were stored in a refrigerator at 1-4°C in liquid state.

3.2.3 Culture media

The seeds of the P. cineraria were germinated on a medium containing 0.8% agar and 1.0% sucrose. The various media used for the culture are described in the table 2. All the media contained the Murashige and Skoog's (1962) basal medium or White's Medium (Table 2) and differed only in the kind and concentration of growth hormones. In some of the media 0.3% activated charcoal or 360 mg/litre polyvinylpyrrolidone (PVP) was added. The pH of the medium was adjusted to 5.8-5.9, with the help of 1N HCl and 1N NaOH, before autoclaving. After melting the agar at 1 kg per sq. cm pressure, the media were dispensed either into culture tubes or conical flasks. The media were sterilized as described earlier.

3.2.4 Seed germination

The overnight presoaked seeds were surface sterilized by treating with 90% ethanol for 20 seconds and 0.1% HgCl_2 solution for 10 minutes, followed by three rinses in sterile distilled water. Sterilized seeds were aseptically inoculated on agar - sucrose medium for germination under light and dark cycles of 16/8 h at $25 \pm 1^\circ\text{C}$.

3.2.5 Micropropagation

3.2.5.1 Explant and callus cultures

Cotyledon, hypocotyl, root and shoot tip explants were excised from 7-15 days old seedlings and surface disinfected explants (axillary bud, shoot tip, young

Table 2 Composition of MS (Murashige and Skoog, 1962) and WM (White, 1934) Media

Constituents		MS (mg/l)	WM (mg/l)
A) Inorganic constituents			
Major constituents	NH_4NO_3	1650	-
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	-
	KNO_3	1900	80
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	720
	KH_2PO_4	170	-
	Na_2EDTA	37.3	-
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8	-
	Na_2SO_4	-	200
	KCl	-	65
	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	-	300
	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	-	16.5
Minor constituents	H_3BO_3	6.2	1.5
	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	7
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	3
	KI	0.83	0.75
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	-
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	-
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	-
	$\text{Fe}_2(\text{SO}_4)_3$	-	2.5
B) Organic constituents			
	Myo-inositol	100	-
	Glycine	2.0	3
	Thiamine-HCl	0.1	0.1
	Pyridoxine-HCl	0.5	0.1
	Cystenic-HCl	-	1
	Nicotinic acid	0.5	0.5
	Calcium panthothenate	-	1
	Sucrose	30000	20000
	Agar-Agar	8000	8000
	pH	5.8	5.8

leaves, internodal segments) from juvenile and mature trees were cultured onto different media (Table 3) for callus initiation and/or plant regeneration. The explants were placed horizontally (hypocotyl, root, cotyledon, internodal segments and young leaves) or vertically (shoot-tip and axillary bud) on the culture medium. Each flask contained 5 explants and one in each test-tube. About 50 explants were cultured per variation of medium/explant type. Initially seedling explants were placed on different media for callus/organogenetic response. Explants from juvenile and mature tree were cultured on media which gave better response with seedling explants.

The cultures were assessed for the frequency of explants regenerating shoots, number of shoots per regenerating explant and callus and root formation periodically for two months. Experiments were repeated 2 times. The cultures were regularly subcultured on fresh medium whenever required. Callus initiated on the cultured explants was separated and recultured on the same media.

3.2.5.2 Rooting

Some of the shoots showed rooting in the parent medium itself. In other cases, regenerated shoots were individually cultured on MS/half strength MS/White's medium containing either different concentrations of auxins (IAA, NAA, IBA) or combination of auxin (IAA, NAA, IBA) and cytokinins (Kn, BA) (Table 4). In some of the media 0.2%/0.3% activated charcoal or 360 mg/litre PVP was also added. The cultures were assessed for root differentiation after 35 days of incubation.

- All the cultures were kept under fluorescent light (4000 lux intensity) / dark cycles of 16/8 h at $25 \pm 1^\circ\text{C}$.

Table 3 List of media tried for callus induction/shoot regeneration in different explants of Prosopis cineraria

Medium code	Growth regulators		Activated charcoal (gm/l)	PVP (mg/l)
	Auxin (mg/l).	Cytokinin (mg/l)		
HC-1	-	-	-	-
HC-2	IBA 3.0	KIN 0.05	-	-
HC-2 PVP	IBA 3.0	KIN 0.05	-	360
HC-3	NAA 4.0	KIN 0.05	-	-
HC-3 PVP	NAA 4.0	KIN 0.05	-	360
HC-4	NAA 5.0	KIN 0.05	-	-
HC-5	NAA 6.0	KIN 0.05	-	-
HC-6 PVP	IAA 6.0	KIN 0.05	-	360
HC-7	-	KIN 0.02	-	-
HC-8	IAA 2.0	KIN 0.2	-	-
HC-9 AC	IAA 3.0	KIN 0.2	3	-
HC-10	NAA 0.2	KIN 0.5	-	-
HC-10 PVP	NAA 0.2	KIN 0.5	-	360
HC-11	IAA 1.0	KIN 0.5	-	-
HC-12	2,4-D 2.0	KIN 0.5	-	-
HC-13	IBA 0.2	KIN 0.5	-	-
HC-14	IBA 2.0	KIN 2.0	-	-
HC-15	IAA 0.25	KIN 4.5	-	-
HC-15 PVP	IAA 0.25	KIN 4.5	-	360
HC-16	IAA 1.0	KIN 5.0	-	-
HC-17 AC	IAA 2.0	KIN 6.0	3	-
HC-18	IAA 0.2	ZEA 0.05	-	-

contd.....

Table 3 contd.....

Medium code	Growth regulators		Activated charcoal (gm/l)	PVP (mg/l)
	Auxin (mg/l)	Cytokinin (mg/l)		
HC-19	IAA 0.5	ZEA 0.05 BA 2.0	-	-
HC-20	NAA 0.2	ZEA 0.5	-	-
HC-20 PVP	NAA 0.2	ZEA 0.5	-	360
HC-20 AC	NAA 0.2	ZEA 0.5	3	-
HC-21	IAA 0.18	BA 0.23	-	-
HC-22 PVP	IAA 0.2	BA 0.5	-	360
HC-23	IBA 0.2	BA 0.5	-	-
HC-24	NAA 0.2	BA 0.5	-	-
HC-25 PVP	NAA 0.05	BA 1.0	-	360
HC-26	2,4-D 1.0 NAA 1.0	BA 1.0	-	-

* All the media contained Murashige and Skoog (1962) basal medium.

** Polyvinylpyrrolidone (PVP), 360 mg/l.

*** Activated charcoal (AC), 3 g/l.

Table 4 Root induction media for cultured P. cineraria shoots

Medium code	Basal Medium	Growth regulators		Activated charcoal (gm/l)	PVP (mg/l)	Agar (gm/l)
		Auxin (mg/l)	Cytokinin (mg/l)			
HCR-1	MS	IBA 3.0	-	2	-	8
HCR-2	MS	IBA 3.0	KIN 0.04	-	-	8
HCR-3	MS	IBA 3.0	KIN 0.05	-	360	6
HCR-4	MS	IBA 4.0	-	-	360	8
HCR-5	MS	IBA 4.0	KIN 0.05	3	-	8
HCR-6	MS	IAA 0.2	-	-	-	8
HCR-7	Half MS	IAA 2.0 NAA 2.0	-	-	-	8
HCR-8	MS	IAA 6.0	KIN 0.05	-	360	8
HCR-9	MS	NAA 0.5	BA 1.0	3	-	8
HCR-10	WM	IBA 3.0	KIN 0.04	-	-	8

3.2.5.3 Transfer of plants from test tube to pots

Once the roots had developed, plantlets were taken out of the culture vessels along with medium and washed in running tap water. Regenerated plants were transferred to half strength MS basal medium/tap water for acclimatization. Thereafter plantlets were transferred to small pots containing a sterile soil : vermiculite mixture (3:1, v/v) and covered initially with beakers to maintain humidity. The beakers were removed from pots periodically for fresh aeration. Upon the first sign of leaf proliferation, plants were taken to a net house. Data were recorded for the percentage success of transfer of plants from test tubes to pots.

3.2.5.4 Statistical analysis

The effect of different media for a particular explant was studied by fitting the following linear model:

$$Y_{ij} = \mu + m_i + e_{ij}$$

where

Y_{ij} = the %response in j^{th} replication of i^{th} medium,

μ = the population mean

m_i = the fixed effect of i^{th} medium

e_{ij} is a random error normally and independently distributed with mean zero and same but unknown variance σ^2_e

The effect of media and explants on percentage response in terms of shoot regeneration or callus induction were studied by fitting the following linear model:

$$Y_{ijk} = \mu + m_i + p_j + (mp)_{ij} + e_{ijk}$$

where

Y_{ijk} = percentage response in k^{th} replication in i^{th} medium and j^{th} explants

μ = the over all mean

m_i = the fixed effect of i^{th} medium

p_j = the fixed effect of j^{th} explant

$(mp)_{ij}$ = the fixed effect of interaction of i^{th} media and j^{th} explant.

e_{ijk} is a random error normally and independently distributed with mean zero and same but unknown variance σ_e^2 .

Data being in percentage were converted into Arc sin value before performing the statistical analysis.

Critical differences were calculated by using standard formula:

$$\text{C.D.} = t \times \text{S.E.m} \times \sqrt{2}$$

where

t is the table value of t - distribution at error degree of freedom and 5% level of significance.

S.E.m = Standard of error of mean.

EXPERIMENTAL RESULTS

To develop efficient protocols for plant regeneration in Prosopis cineraria from seedlings, juvenile and mature tree explants, various media combinations were tried during present investigation. These protocols are necessary for genetic manipulation and tree improvement in this species.

The observations recorded during the course of investigation are presented below.

4.1. In vitro response of various explants in different media

4.1.1 Callus induction

Table 5 to 12 describe the effect of different hormonal combinations and other additives in the media on callus induction in different explants of P. cineraria.

4.1.1.1 *Callus response of seedling explants in different media*

A. Hypocotyl: Callus induction was observed in hypocotyl explants on most of the

media tried. Initiation of callus took place within 10-15 days of inoculation. Callus formation was seen usually at the cut end and callus was creamish yellow (Figs. 1&2). Browning of the callus started 15-20 days after callus initiation. Further subculturing of callus on the same medium did not favour differentiation. Maximum per cent response (95%) for callusing was observed on medium containing NAA (0.2 mg/l) and ZEA (0.5 mg/l) with or without activated charcoal, but addition of PVP has significantly reduced the response (Table 5). For hypocotyl explants, when BA was used, it resulted in low or no response and Zeatin was the best supplement in the media followed by KIN and BA.

B. Root: In root segments, callus initiation was observed in 12-15 days of culturing. Callus developed on the entire surface and was initially soft and white (Figs 3&4), but turned brown on subsequent culturing and failed to show any morphogenetic response thereafter. Maximum callus induction (65%) was observed on medium (HC-20) supplemented with ZEA (0.5 mg/l) and NAA (0.2 mg/l), although it was not significantly different from the media HC-3 and HC-15. For root explants also addition of BA in the media resulted in no or poor callusing (Table 6). In HC-2, HC-3 and HC-15 media roots were also observed along with callus formation. However, on HC-5 medium only roots were developed.

C. Cotyledon: Initiation of callus took place within 10-12 days of inoculation in cotyledonary explants. Callusing was observed at the outer end of the petiole (Fig. 5). Cotyledon explants developed light brown callus with limited growth. It turned brown after 20 days of initiation and did not show further response. Callusing was observed only on three media HC-10 PVP, HC-15 PVP and HC-22 PVP and maximum percentage of callusing (35.4%) was observed on HC-22 PVP medium but was not significantly different from HC-10 PVP medium (Table 7).

Fig. 1 Callus formation in hypocotyl segments of
P. cineraria on HC-20 medium.

Fig. 2 Enlarged view of callusing in hypocotyl
segment.

Fig. 3 Root callus on HC-15 medium.

Fig. 4 Enlarged view of initiated root callus.

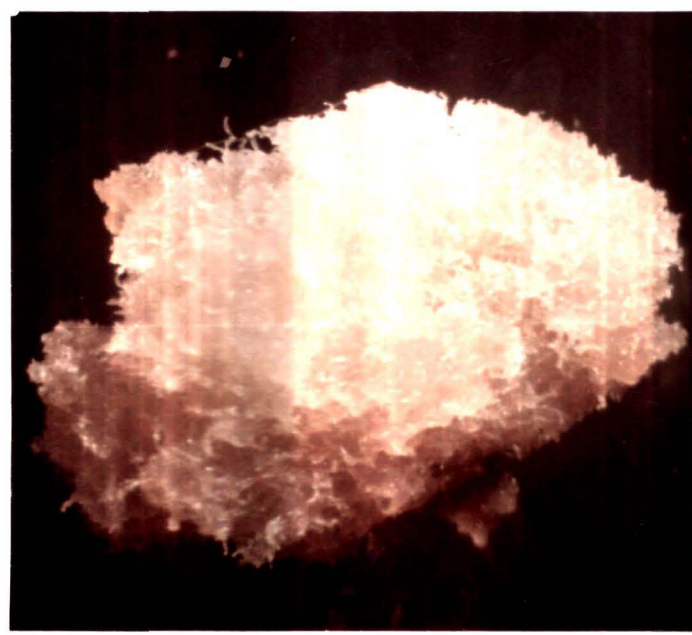
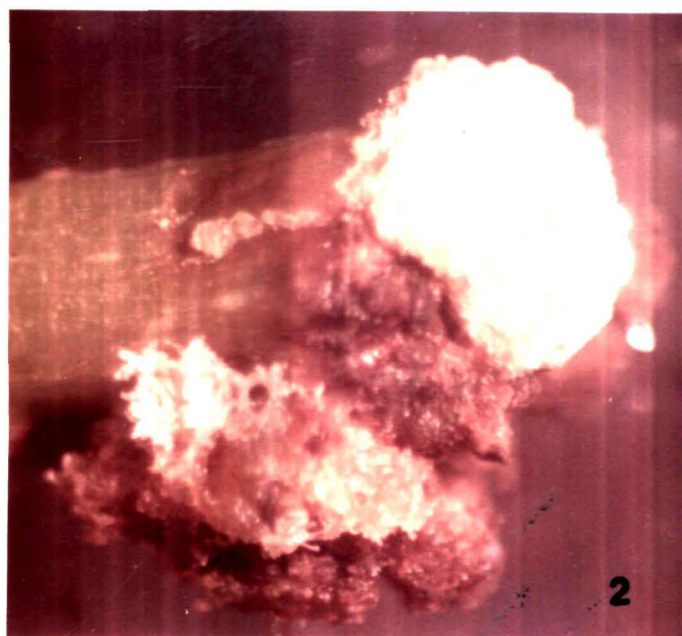


Table 5 Response of *P. cineraria* hypocotyl segments on different media

Medium code	Growth regulators				Number of explants cultured	Mean response*	
		Auxin (mg/l)	Cytokinin (mg/l)			Callus	Shoot
**HC-2	IBA	3.0	KIN 0.05		48	35.8 ^e (34.3±5.71)	
HC-3	NAA	4.0	0.05		45	26.1 ^{cd} (19.5±4.5)	0
HC-4		5.0	0.05		40	42.1 ^{efgh} (45.0±5.0)	0
HC-5		6.0	0.05		48	40.1 ^{efgh} (41.4±1.4)	0
HC-6 PVP	IAA	6.0	0.05		50	14.0 ^{ab} (06.0±2.0)	0
HC-10	NAA	0.2	0.5		50	48.0 ^{gh} (55.0±5.0)	0
HC-10 PVP		0.2	0.5		50	50.8 ^h (60.0±0.0)	0
HC-11	IAA	1.0	0.5		50	34.3 ^{de} (32.0±8.0)	9.7 ⁿ (4.0±4.0)
HC-12	2,4-D	2.0	0.5		63	49.2 ^h (57.3±7.3)	0
HC-13	IBA	0.2	0.5		50	36.8 ^{ef} (36.0±4.0)	0
HC-15	IAA	0.25	4.5		46	45.7 ^{fgh} (51.2±8.9)	0
HC-15 PVP		0.25	4.5		73	49.1 ^{gh} (57.0±5.5)	26.5 ^a (20.1±4.9)
HC-16		1.0	5.0		40	0	0
HC-18		0.2	ZEA 0.05		40	0	0
HC-19		0.5	ZEA 0.05 BA 2.0		60	21.3 ^{abc} (13.3±3.3)	0

contd.....

Table 5 contd.....

Medium code		Growth regulators			Number of explants cultured	Mean response*	
		Auxin (mg/l)	Cytokinin (mg/l)			Callus	Shoot
HC-20	NAA	0.2	ZEA	0.5	60	77.5 ⁱ (95.1±0.9)	0
HC-20	PVP	0.2		0.5	40	63.4 (80.0±0.0)	0
HC-20	AC	0.2		0.5	40	76.6 ⁱ (94.5±1.5)	13.3 ^a (5.3±1.3)
HC-21	IAA	0.18	BA	0.23	35	0	0
HC-22	PVP	0.2		0.5	45	22.5 ^{bc} (15.0±5.0)	0
HC-23	IBA	0.2		0.5	40	0	0
HC-24	NAA	0.2		0.5	66	12.3 ^a (4.7±1.9)	0
HC-26	2,4-D	1.0		1.0	45	0	0
	NAA	1.0					
C.D. value at P≤0.05						9.0	20.4

* Mean response represents transformed values of per cent response,

Values in bracket show mean percent response with standard error.

** Percentage rooting = 7.90 ± 2.9

Note: Means having the same subscript are not significantly different.

Table 6 Response of *P. cineraria* root segments on different media

Medium code	Growth regulators				Number of explants cultured	Mean response*		
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot	Root
HC-2	IBA	3.0	KIN	0.05	50	33.2 ^{abc} (30±2)	0	17.6 ^u (10±6)
HC-3	NAA	4.0		0.05	45	46.7 ^{de} (53.±3)	0	19.8 ^a (12.5±7.5)
HC-4		5.0		0.05	50	39.2 ^{bcd} (40±8)	0	0
HC-5		6.0		0.05	50	0	0	27.8 ^a (22±6)
HC-6 PVP	IAA	6.0		0.05	45	26.8 ^{abc} (20.5±4.5)	0	0
HC-10	NAA	0.2		0.5	45	39.7 ^{cd} (41.±9)	0	0
HC-10 PVP		0.2		0.5	40	33.1 ^{abc} (30±5)	25.2 ^a (19±9)	0
HC-11	IAA	1.0		0.5	47	25.9 ^{ab} (19.1±0.9)	0	0
HC-12	2,4-D	2.0		0.5	50	39.2 ^{bcd} (40±8)	0	0
HC-13	IBA	0.2		0.5	45	0	0	0
HC-15	IAA	0.25		4.5	80	51.0 ^{de} (60.3±6.3)	0	21.3 ^a (13.3±3.3)
HC-15 PVP		0.25		4.5	40	37.8 ^{abcd} (37.5±2.5)	16.5 ^a (8.7±4.7)	0
HC-16		1.0		5.0	50	25.4 ^a (20±12)	0	0
HC-18		0.2	ZEA	0.05	40	0	0	0
HC-19		0.5	ZEA BA	0.05 2.0	50	0	0	0

Table 6 contd.....

Table 6 contd.....

Medium code	Growth regulators				Number of explants cultured	Mean response*		
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot	Root
HC-20	NAA 0.2		ZEA 0.5		60	53.8 ^e (65±5)	0	0
HC-20 PVP		0.2		0.5	50	39.2 ^{bed} (40±8)	0	0
HC-20 AC		0.2		0.5	25	0	17.0 ^a (13.3±13.3)	0
H-21	IAA 0.18		BA 0.23		50	0	0	0
HC-22 PVP		0.2		0.5	46	24.8 ^a (17.7±2.3)	0	0
HC-23	IBA 0.2			0.5	40	0	0	0
HC-24	NAA 0.2			0.5	50	0	0	0
HC-26	2,4-D 1.0 NAA 1.0			1.0	40	0	0	0
C.D. value at $P \leq 0.05$						13.3	42.7	20.4

* Mean response represents transformed values of per cent response,

Values in brackets show mean per cent response with standard error.

Note: Means having the same subscript are not significantly different.

Table 7 Callus induction and plant regeneration from cotyledonary explants of P. cineraria on different media

Medium Code	Growth regulators				Number of explants cultured	Mean response*	
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot
HC-1	-		-		48	0	16.9 ^a (8.6±1.4)
HC-2	IBA	3.0	KIN	0.05	45	0	0
HC-3	NAA	4.0		0.05	45	0	0
HC-4		5.0		0.05	40	0	0
HC-5		6.0		0.05	40	0	0
**HC-6 PVP	IAA	6.0		0.05	48	0	44.6 ^{fg} (9.3±4.3)
HC-10 PVP	NAA	0.2		0.5	44	22.8 ^{ab} (15.4±5.4)	30.9 ^{cd} (26.7±6.7)
HC-11	IAA	1.0		0.5	56	0	39.1 ^{def} (39.7±4.3)
HC-12	2,4-D	2.0		0.5	50	0	0
HC-13	IBA	0.2		0.5	56	0	15.6 ^a (7.2±0.8)
HC-15 PVP	IAA	0.25		4.5	50	21.9 ^a (14±2)	49.0 ^g (58±2)
HC-16		1.0		5.0	45	0	0
HC-18		0.2	ZEA	0.05	45	0	14.7 ^a (6.5±1.5)
HC-19		0.5	ZEA BA	0.05 2.0	48	0	41.3 ^{efg} (43.6±4.4)
HC-20	NAA	0.2	ZEA	0.5	50	0	21.5 ^{ab} (14±6)
HC-20AC		0.2		0.5	30	0	28.0 ^{bc} (22.5±7.5)
HC-21	IAA	0.18	BA	0.23	50	0	0

(Contd.....7

Table 7 contd.....

Medium code	Growth regulators		Number of explant cultured	Mean response*	
	Auxin (mg/l)	Cytokinin (mg/l)		Callus	Shoot
HC-22 PVP	IAA 0.2	0.5	56	36.5 ^b (35.4±4.6)	32.2 ^{cde} (28.5±1.5)
HC-23	IBA 0.2	0.5	60	0	26.3 ^{bc} (19.9±6.7)
HC-24	NAA 0.2	0.5	40	0	30.0 ^{bcd} (25±0)
HC-26	2,4-D 1.0 NAA 1.0	1.0	40	0	0
C.D. value at $P \leq 0.05$				14.1	9.3

* Mean response represents transformed values of per cent response,
Values in bracket show mean per cent response with standard error.

** Percentage rooting = 20.7 ± 0.7 .

Note: Mean having the same subscript are not significantly different.

D. Shoot tip: In shoot tip explants no callusing was observed on all the media tried (Table 8).

4.1.1.2 Callus response of juvenile tree explants in different media

A. Internodal segment: In case of internodal explants excised from juvenile tree, maximum percentage of callusing (62.4%) was obtained on HC-22 PVP medium supplemented with IAA (0.2 mg/l) and BA (0.5 mg/l) although it did not differ significantly from HC-15 PVP and HC-17 AC medium (Table 9). When low cytokinin and high auxin is added in the medium then there is no or very poor callus induction. Initiation of callusing was observed in 11-15 days of inoculation but further growth of callus remained slow or arrested. Callusing was observed all over the explant and it was light brown in colour (Fig. 6). It failed to show any morphogenetic response.

B. Axillary bud: In axillary bud excised from juvenile tree, callusing was observed 10-12 days after culturing on most of the media tried. Callus was initiated at the node and was white or light brown in colour. Maximum frequency of callusing (69.7%) was observed on HC-17 AC medium supplemented with IAA (2.0 mg/l) and KIN (6.0 mg/l). Relatively good callusing was observed on HC-15 PVP, HC-10 PVP, HC-22 PVP and HC-20 AC media and there was no or poor response on medium containing high auxin and low Kinetin (Table 10).

4.1.1.3 Callus response of mature tree explants in different media

A. Internodal segment: In internodal segments excised from mature tree, the callus initiated in 10-15 days. The callus was white and its growth was limited. Callusing was observed all over the explant. Callus turned brown in 30 days after its initiation (Fig. 7) and did not show further response. Maximum percentage of callusing (50%) was observed on medium (HC-22 PVP) containing 0.2 mg/l IAA

- Fig. 5** Callus induction in cotyledonary explants of P. cineraria on HC-10 PVP medium.
- Fig. 6** Callusing in internodal segment of juvenile P. cineraria on HC-17 AC medium.
- Fig. 7** Callus formation in internodal segment of mature P. cineraria on HC-17 AC medium.

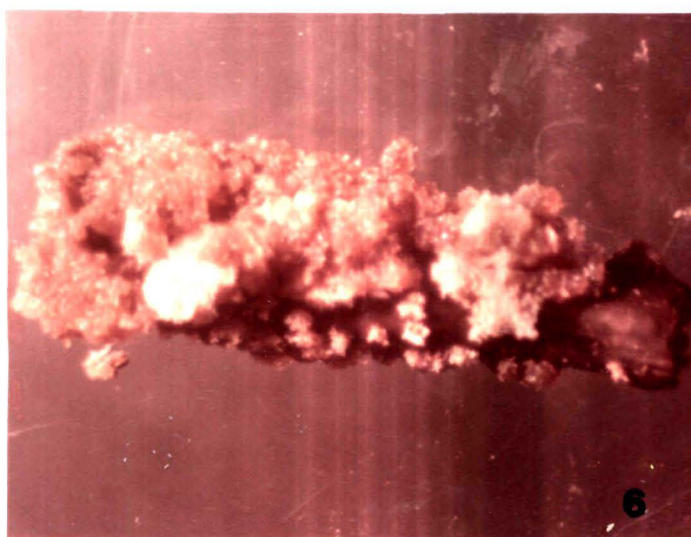
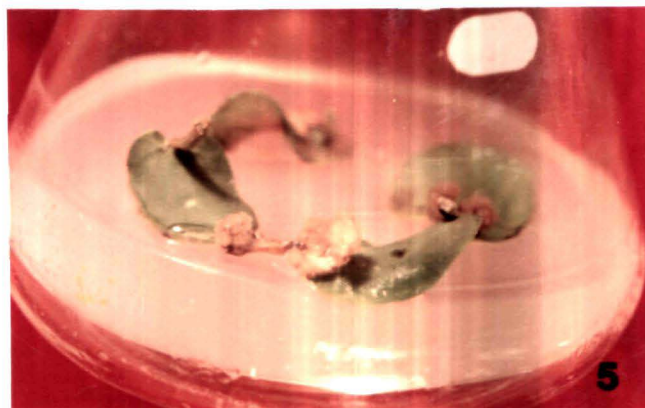


Table 8 Callus induction and shoot regeneration from seedling shoot tip explants of P cineraria on different media

Media code	Growth regulators				Number of explants cultured	Mean response*	
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot
HC-1	-		-		40	0	23.4 ^{bc} (16±4)
HC-2	IBA	3.0	KIN	0.05	30	0	8.9 ^a (3.3±3.3)
HC-6 PVP	IAA	6.0		0.05	48	0	44.9 ^d (49.7±6.3)
HC-10 PVP	NAA	0.2		0.5	45	0	19.4 ^{ab} (11±1)
HC-11	IAA	1.0		0.5	35	0	23.4 ^{bc} (16±4)
HC-13	IBA	0.2		0.5	50	0	14.0 ^{ab} (6±2)
HC-15 PVP	IAA	0.25		4.5	52	0	42.7 ^d (45.9±5.9)
HC-20 AC	NAA	0.2	ZEA	0.5	20	0	45.0 ^d (50±10)
HC-22 PVP	IAA	0.2	BA	0.5	50	0	33.1 ^{cd} (30±6)
C.D. values at P≤ 0.05							12.4

* Mean response represents transformed values of per cent response,
Values in bracket show mean per cent response with standard error.

Note: Mean having the same subscript are not significantly different.

Table 9 Callus induction and plant regeneration in internodal explants of juvenile P. cineraria on different media

Medium code	Growth regulators				Number of explants responding	Mean response*	
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot
HC-1	-		-		40	0	0
HC-2 PVP	IBA	3.0	KIN	0.05	52	8.7 (3.1±3.1)	0
HC-6 PVP	IAA	6.0		0.05	45	0	0
HC-7	-			0.2	35	0	0
HC-8	IAA	2.0		0.2	40	0	0
HC-10 PVP	NAA	0.2		0.5	60	33.1 ^a (30±3.4)	0
HC-15 PVP	IAA	0.25		4.5	45	46.9 ^{bc} (53.4±1.2)	0
HC-17 AC		2.0		6.0	64	39.6 ^{abc} (40.6±0.6)	0
HC-20 AC	NAA	0.2	ZEA	0.5	45	35.7 ^{ab} (34.5±10.5)	0
HC-22 PVP	IAA	0.2	BA	0.5	48	52.2 ^c (62.4±1.6)	0
C.D. value at P≤0.05						12.7	

* Mean response represents transformed values of per cent response,
Values in bracket show mean per cent response with standard error.

Note: Means having the same subscript are not significantly different.

Table 10 In vitro response of axillary buds of juvenile P. cineraria on different media

Medium code	Growth regulators				Number of explants cultured	Mean response*	
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot
HC-1	-		-		35	0	0
HC-2 PVP	IBA	3.0	KIN	0.05	43	7.4 ^a 2.3	7.9 (2.5±2.5)
HC-6 PVP	IAA	6.0		0.05	47	7.6 ^a (2.3±2.3)	20.5 ^a (12.5±3.5)
HC-7	-			0.2	40	0	0
HC-8	IAA	2.0		0.2	40	0	0
HC-9 AC		3.0		0.2	50	0	7.2 (2.0±2.0)
HC-10 PVP	NAA	0.2		0.5	56	20.6 ^b (12.4±0.9)	36.5 ^b (35.4±4.6)
HC-15 PVP	IAA	0.25		4.5	50	15.9 ^{ab} (8.0±4.0)	33.1 ^b (30.0±6.0)
HC-17 AC		2.0		6.0	52	56.6 (69.7±3.0)	28.7 ^{ab} (23.0±0.3)
HC-20 AC	NAA	0.2	ZEA	0.5	45	27.8 ^b (22.0±2.0)	28.3 ^{ab} (22.5±2.5)
HC-22 PVP	IAA	0.2	BA	0.5	43	26.9 ^b (21±7.6)	38.0 ^b (37.9±2.1)
C.D. value at P≤0.05						12.5	10.3

* Mean response represents transformed values of per cent response,
Values in bracket show mean per cent response with standard error.

Note: Means having the same subscript are not significantly different.

and 0.5 mg/l BA although percent response on HC-10 PVP, HC-20 AC, HC-17 AC and HC-15 PVP was not significantly different. Low KIN and high auxin in the medium results in no or very low response of callus induction (Table 11).

B. Axillary bud: Axillary bud of mature tree showed callus initiation with in 10-12 days of culturing. Callusing was observed only on three media and highest percentage of callusing (65.8%) was observed on HC-22 PVP medium supplemented with IAA (0.2 mg/l) and BA (0.5 mg/l) (Table 12). Callusing was seen at the node and it was white or light brown. The callus developed from explants failed to grow and turned brown on same or different media after first subculture. Therefore, all attempts to maintain the callus obtained from various explants failed.

C. In shoot tip and young leaves of juvenile and adult tree no callusing was observed.

Analysis of variance for response of individual explants for callus induction in different media revealed highly significant ($P \leq 0.01$) differences among the media for callus induction in all the explants except cotyledon where the differences among the media were found non-significant.

Performance of five promising media namely, HC-6 PVP, HC-10 PVP, HC-15 PVP, HC-20 AC and HC-22 PVP were examined for callus induction in various explants. Only the explants for which response was observed were considered for analysis of variance test. Analysis of variance was performed to test the main effects of media and explants along with interaction between them. All the three effects were found highly significant for callusing (Table 13). Further the differences among explants in a particular medium were tested by the critical difference test. These are presented in Table 14.

On HC-6 PVP medium which contained IAA (6.0 mg/l) and KIN (0.5 mg/l) response of root explants is highest (20.5%) but it is not significantly different

Table 11 Callus induction and plant regeneration in internodal explants of *P. cineraria* on different media

Medium code	Growth regulators		Number of extplants cultured	Mean response*	
	Auxin (mg/l)	Cytokinin (mg/l)		Callus	Shoot
HC-1	-	-	45	0	0
HC-2-PVP	IBA 3.0	KIN 0.05	45	0	0
HC-3 PVP	NAA 4.0	0.05	40	0	0
HC-4	NAA 5.0	0.05	45	0	0
HC-6 PVP	IAA 6.0	0.05	45	19.3 ^a (11.1±2.0)	0
HC-7	-	0.2	40	0	0
HC-8	IAA 2.0	0.2	35	0	0
HC-9 AC	3.0	0.2	48	9.2 ^a (3.8±3.6)	0
HC-10 PVP	NAA 0.2	0.5	58	33.6 ^b (30.8±5.8)	0
HC-15 PVP	IAA 0.25	4.5	48	40.0 ^b (41.4±6.6)	0
HC-17 AC	2.0	6.0	60	35.5 ^b (33.7±2.3)	0
HC-20 AC	NAA 0.2	ZEA 0.5	45	34.6 ^b (32.5±7.5)	0
HC-22 PVP	IAA 0.2	BA 0.5	50	45.0 ^b (50±2.0)	0
C.D. values at $P \leq 0.05$				12.4	

* Mean response represents transformed values of mean per cent response.
Values in bracket show mean per cent response with standard error.

Note: Means having the same subscript are not significantly different.

Table 12 Callus induction and plant regeneration from axillary bud of mature *P. cineraria*

Me dium code	Growth regulators				Number of explants cultured	Mean response*	
	Auxin (mg/l)		Cytokinin (mg/l)			Callus	Shoot
HC-1	-		-		45	0	0
HC-2 PVP	IBA	3.0	KIN	0.05	45	0	7.2 ^a (2.0±2)
HC-3 PVP	NAA	4.0		0.05	40	0	0
HC-4	NAA	5.0		0.05	55	0	0
HC-6 PVP	IAA	6.0		0.05	48	0	19.1 ^{abc} (11.0±3.9)
HC-7	-			0.2	40	0	0
HC-8	IAA	2.0		0.2	45	0	0
HC-9 AC		3.0		0.2	50	0	7.2 ^a (2±2)
HC-10 PVP	NAA	0.2		0.5	45	0	26.7 ^{bc} (21±9)
HC-15 PVP	IAA	0.25		4.5	73	15.2 ^a (6.9±1.7)	35.6 ^c (34.0±5.5)
HC-20 AC	NAA	0.2	ZEA	0.5	48	16.0 ^a (7.9±2.9)	30.7 ^{bc} (27.1±12.9)
HC-22 PVP	IAA	0.2	BA	0.5	67	53.9 (65.8±0.9)	26.3 ^{bc} (20.0±6.6)
HC-25 PVP	NAA	0.05		1.0	64	0	15.3 ^{ab} (7.5±4.2)
C.D. values at P≤ 0.05						9.4	17.2

* Mean response represents transformed values of per cent response,

Values in bracket show mean per cent response with standard error.

Note: Means having the same subscript are not significantly different.

Table 13 Analysis of variance for shoot regeneration/callus induction in different explants of P. cineraria

Sources of variation	Callus		Shoot regeneration	
	D.F.	M.S.	D.F.	M.S.
Between media	4	1394.49**	4	280.90**
Between explants	6	960.61**	5	1421.94**
Media x explants	24	428.20**	20	143.15**
Error	35	15.89	30	40.50

** $P \leq 0.01$

Table 14 Response of different explants on five promising media for callus induction in P. cineraria

Medium code Explant	HC-6 PVP	Hc-10 PVP	HC-15 PVP	HC-20 AC	HC-22 PVP
Root	*27.0 ^d (20.5±4.5)	33.1 ^b (30±5)	37.8 ^b (37.5±2.5)	2.9 ^a (0)	24.8 ^{ab} (17.7±2.3)
Hypocotyl	14.0 ^{bc} (6±2)	50.8 (60±0)	50.8 ^d (57±5.5)	76.6 (94.5±1.5)	22.5 ^a (15±5)
Cotyledon	2.9 ^a (0)	22.8 ^a (15.4±5.4)	21.9 ^a (14±2)	2.9 ^a (0)	36.5 (35.4±4.6)
Internodal segment (Juvenile tree)	2.9 ^a (0)	33.1 ^b (30±3.4)	46.9 ^{cd} (53.4±1.2)	35.7 ^{bc} (34.5±10.5)	52.2 ^{cd} (62.4±1.6)
Axillary bud (Juvenile tree)	7.6 ^{ab} (2.3±2.3)	20.6 ^a (12.4±0.9)	15.9 ^a (8±4)	28 ^b (22±2)	26.9 ^{ab} (21±7.6)
Internodal segment (Mature tree)	19.3 ^{cd} (11.1±2)	33.6 ^b (30.8±5.8)	40 ^{bc} (41.4±6.6)	34.6 ^{bc} (32.5±7.5)	45 ^c (50±2.0)
Axillary bud (Mature tree)	2.9 ^a (0)	2.9 (0)	15.2 ^a (6.9±1.7)	16.0 (7.9±2.9)	53.9 ^d (65.8±0.9)

C.D. value at $P \leq 0.05 = 8.1$

* Values in the bracket are mean per cent response with standard error and values above bracket are Arc Sin-value of per cent response.

Note: Means having the same subscript are not significantly different.

from response of internodal segments of mature tree. Lowest percentage of callusing was observed in internodal segment and axillary bud of juvenile tree and cotyledonary explants.

In HC-10 PVP and HC-15 PVP medium, maximum percentage of callusing (60% and 57% respectively) was observed in hypocotyl explants and lowest response was obtained in axillary bud excised from mature tree. Callus response in root and internodal explants of juvenile and mature tree is relatively good and is not significantly different.

In case of medium (HC-20 AC) containing NAA (0.2 mg/l) and ZEA (0.5 mg/l) maximum frequency of callusing (94.5%) was found in hypocotyl explants.

On HC-22 PVP medium performance of axillary bud excised from mature tree was found to be highest (65.8%) but there was no significant difference (62.4%) in internodal segment of juvenile tree. Poor response was observed in hypocotyl and root explants and axillary bud of juvenile tree.

4.1.2 Shoot regeneration

Shoot regeneration was achieved by the initiation of adventitious buds and the enhancement of the development of axillary buds and shoot tips. Response of seedling, juvenile and mature tree explants for shoot regeneration on different media are presented in Table 5 to 12.

4.1.2.1 Shoot regeneration from seedling explants in different media

A. Hypocotyl: In hypocotyl segments, shoot bud initiation was observed within 50-60 days of growth and well developed shoots were observed after 3 month growth on HC-11, HC-15 PVP and HC-20 AC medium (Fig. 8). The differentiated shoots were about 3-5 cms in length and possessed small leaves and vegetative buds

and no leaf dropping was seen. Highest regeneration frequency (20%) was observed on medium (HC-15 PVP) containing low IAA (0.25 mg/l) and high KIN (4.5 mg/l) (Table 5).

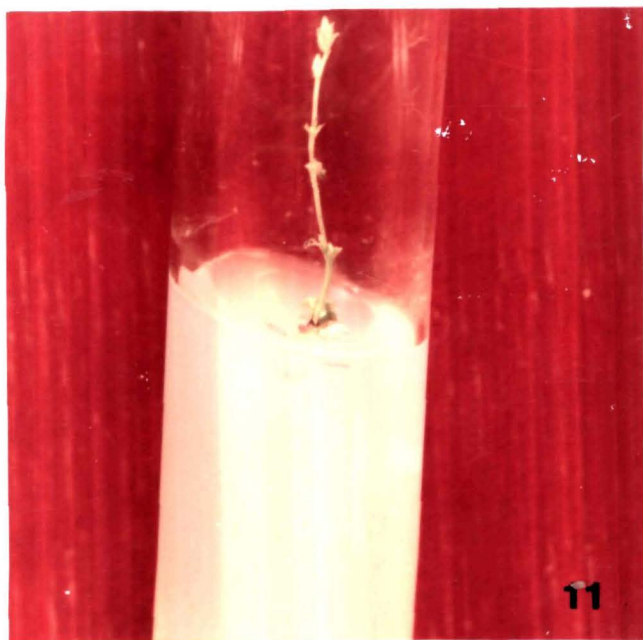
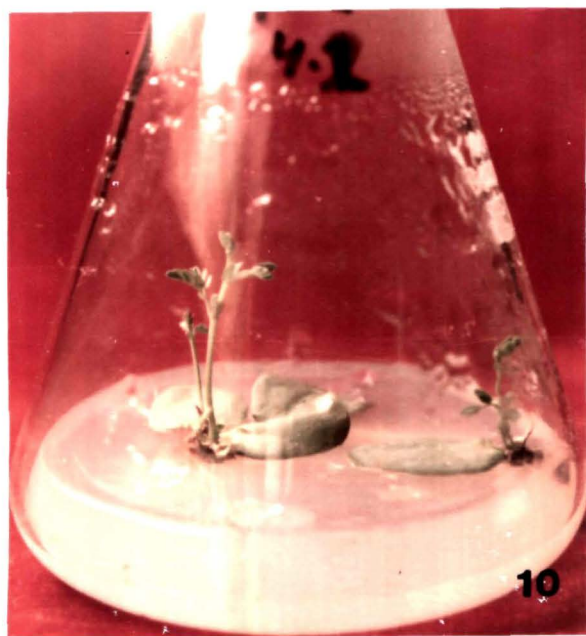
B. Root: Shoot regeneration in case of root segments was observed only on three media namely HC-10 PVP, HC-15 PVP and HC-20 AC and regeneration frequency was also low (Table 6 and Fig. 9). Maximum frequency of regeneration (19%) was obtained on HC-10 PVP medium containing NAA (0.2 mg/l) and KIN (0.5 mg/l).

C. Cotyledon: In cotyledonary explants, initiation of adventitious buds was observed within 10-15 days of incubation. Highest regeneration frequency (58%) was realised on medium HC-15 PVP containing low IAA (0.25 mg/l) and high KIN (4.5 mg/l) using cotyledon as explant (Table 7 and Fig. 10). It can be added that per cent response on HC-19, HC-6 PVP and HC-15 PVP medium was not significantly different. Twenty per cent of the cotyledonary explants cultured on HC-6 PVP medium directly gave rise to both the root and shoot.

D. Shoot tip: Shoot regeneration from seedling shoot tip explants was observed on all the media tried, however, multiple shoots could not be recovered on any of the media. Shoot tip explants that were 1.0-1.5 cms in length regenerated shoots (Fig. 11) but small explants failed to regenerate and died. Maximum frequency of shoot regeneration (50%) was observed on medium HC-20 AC containing NAA (0.2 mg/l) and ZEA (0.5 mg/l) (Table 8). However, shoot morphogenesis on HC-20 AC was not significantly different from HC-6, HC-15 PVP and HC-22 PVP medium. Medium containing IBA gave the poorest results for shoot regeneration.

4.1.2.2 Shoot regeneration from juvenile and mature tree explants in different media

- Fig. 8** Hypocotyl segments of P. cineraria showing development of multiple shoots on HC-11 medium.
- Fig. 9** Root explant of P. cineraria showing shoot bud initiation on HC-20 AC medium.
- Fig. 10** Multiple shoot formation in cotyledonary explants of P. cineraria on HC-15 PVP medium.
- Fig. 11** Shoot regeneration from seedling shoot tip explant of P. cineraria on HC-15 PVP medium.



A. Axillary bud: Shoot buds from axillary bud explants were apparently visible within 4 weeks of culture on several media treatments as shown in Table 10 and 12. In case of axillary bud of juvenile tree shoot regeneration was highest (37.9%, Fig. 12) when the medium contained BA (0.5 mg/l) and IAA (0.2 mg/l), although the per cent shoot regeneration on HC-20 AC, HC-17 AC, HC-15 PVP and HC-10 PVP media was not significantly different from HC-22 PVP medium which contained BA (0.5 mg/l) and IAA (0.2 mg/l) (Table 10 and Fig. 13). In case of axillary bud excised from mature tree, highest regeneration percentage (34%) was realised on HC-15 PVP medium but per cent response on this medium did not differ significantly from response on HC-6 PVP, HC-22 PVP, HC-10 PVP, HC-20 AC and HC-15 PVP media (Table 12, Fig. 14 & 15).

On most of the media, shoot grew to 1.5-2.5 cms in height and after that leaf dropping was observed (Fig. 14). Second and third shoot arise within 15-28 days of appearance of the first shoot. In both the explants (axillary bud of juvenile and mature tree), medium with high auxin and low cytokinin resulted in no or poor shoot regeneration frequency.

B. Internodal segment, leaves and shoot tip explants from juvenile and adult tree remained unresponsive for shoot regeneration under the experimental conditions.

Analysis of variance for response of individual explants for shoot regeneration in different media revealed highly significant/significant differences among the media for shoot regeneration in all the explants except hypocotyl and root explants where differences among the media are non-significant for shoot regeneration.

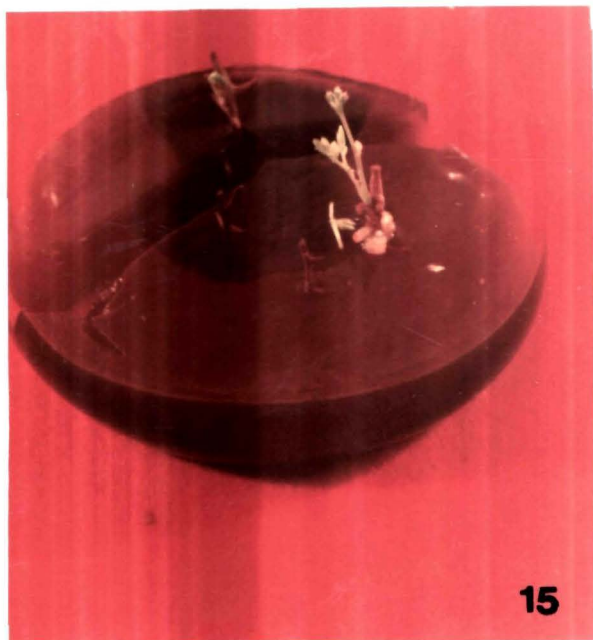
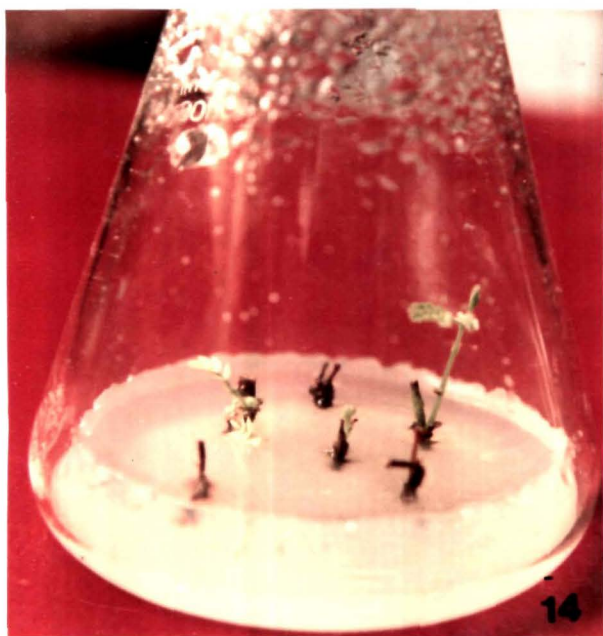
Performance of HC-6 PVP, HC-10 PVP, HC-15 PVP, HC-20 AC and HC-22 PVP were examined for shoot regeneration. Explants for which response was nil in all the five media were dropped from this analysis. Analysis of variance was performed

Fig. 12 Shoot regeneration from axillary buds of juvenile P. cineraria on HC-22 PVP medium.

Fig. 13 Development of axillary shoot from axillary bud explants of juvenile P. cineraria on HC-10 PVP medium.

Fig. 14 Axillary bud explants of mature P. cineraria showing development of shoots on HC-15 PVP medium.

Fig. 15 Axillary bud of mature P. cineraria showing the multiple shoots on HC-20 AC medium.



to test the main effects of media and explants along with interaction between them. All the three effects were found highly significant (Table 13). As the interaction between media and explants was found to be highly significant which indicated that the performance of different explants in a particular media was not uniform and vice-versa. Further the differences among explants for shoot regeneration in a particular medium was tested by the critical difference test (Table 15).

On HC-6 PVP medium response of cotyledonary and shoot tip explants is highest (44.6 and 44.9% respectively) and is not significantly different. No regeneration was obtained from root and hypocotyl explants on this medium. Whereas on medium HC-10 PVP 25.2% shoot regeneration was observed in root segments and was not significantly different from that of cotyledon and axillary bud of juvenile and mature tree. Highest regeneration frequency (35.4%) on HC-10 PVP medium was obtained from axillary bud of juvenile tree.

On medium (HC-15 PVP) performance of cotyledonary explants is best (49%) although it is not significantly different from that of shoot tip explants. Relatively good regeneration frequency was obtained in axillary bud of juvenile and mature tree. In case of HC-20 AC medium shoot regeneration from shoot tip explant is highest (45%) and is lowest (13.3%) in case of hypocotyl segment. Regeneration from cotyledon and axillary bud of juvenile and mature tree is relatively good and is not significantly different.

Maximum frequency of shoot regeneration (37.9%) was obtained from axillary bud of juvenile tree, on HC-22 PVP medium, although it was not significantly different from the per cent response in axillary bud of adult tree, cotyledon and shoot tip explants. No regeneration was observed in case of root and hypocotyl explants.

Table 15 Response of different explants on five promising media for shoot regeneration in P. cineraria

Medium code Explant	HC-6 PVP	HC-10 PVP	HC-15 PVP	HC-20 AC	HC-22 PVP
Root	*2.9 ^a (0)	25.2 ^{ab} (19±9)	16.5 ^a (8.7±4.7)	17 ^{ab} (13.3±13.3)	2.9 (0)
Hypocotyl	2.9 ^a (0)	2.9 (0)	26.5 ^{ab} (20.1±4.9)	13.3 ^a (5.3±1.3)	2.9 (0)
Cotyledon	44.6 ^c (49.3±4.3)	30.9 ^{abcd} (26.7±6.7)	49.0 ^e (58±2)	28.0 ^{bc} (22.5±7.5)	32.1 ^b (28.5±1.5)
Shoot tip	44.9 ^c (49.7±6.3)	19.4 ^a (11±1)	42.7 ^{cde} (45.9±5.9)	45.0 (50±10)	33.1 ^b (30±6)
Axillary bud (Juvenile tree)	20.5 ^b (12.5±3.5)	36.5 ^{bcd} (35.4±4.6)	33.1 ^{bc} (30±6)	28.3 ^{bc} (22.5±2.5)	38 ^b (37.9±2.1)
Axillary bud (Mature tree)	19.1 ^b (11.1±3.9)	26.7 ^{abc} (21±9)	35.6 ^{bcd} (34.0±5.5)	30.7 ^c (27.1±12.9)	26.3 ^b (20.1±6.6)

C.D. value at $P \leq 0.05 = 13.109$

* Values in the bracket are mean per cent response with standard error and values above bracket are Arc Sin value of per cent response.

Note : Means having the same subscript are not significantly different.

4.1.3 Multiple shoot formation

Multiple shoots were observed from hypocotyl, root, cotyledon and axillary bud on media listed in Table 16.

Multiple shoot formation was highly dependent on source of explants as no single medium produced multiple shoots in all the explants under study. Cotyledonary explants produced 1-2 shoots in each explant on HC-10 PVP, HC-20 AC and HC-22 PVP medium and 2-3 shoots on HC-15 PVP medium (Fig. 10). Root explants produced 1-2 shoots per regenerating explant in HC-10 PVP and HC-15 PVP medium. Hypocotyl segments produced 2-3 shoots in HC-11 and HC-20 AC medium while on HC-15 PVP medium 2-4 shoots per explant were observed (Fig. 8). Axillary bud explants produced multiple shoots on HC-22 PVP and HC-20 AC medium (Fig. 15). In rest of the media solitary shoots were developed.

4.1.4 Browning

To prevent browning of explants and medium several methods were tried.

- a) Explants were transplanted into fresh medium whenever browning occurred.
- b) Newly excised explants were soaked for three hours in a solution of antioxidant (400 mg/100 ml citric acid).
- c) Explants were grown on media containing antioxidants.

Browning of explants was observed when explants were soaked in antioxidant solution and then cultured on medium devoid of PVP but this could be overcome by quick subculturing. When explants were not soaked in antioxidant solution before culturing in medium containing PVP (360 mg/l) relatively more browning was observed. However, when a combination of the two i.e. soaking in antioxidant solution as well as culturing in media supplemented with PVP (360 mg/l) was employed, no browning of the medium was observed.

Table 16 Shoot regeneration from different explants of P. cineraria on a few promising culture media

Medium code	Growth regulators				Explants	Number of shoots/ regenerating explant
	Cytokinin (mg/l)		Auxin (mg/l)			
HC-10 PVP	KIN	0.5	NAA	0.2	root	1-2
					axillary bud	1
					cotyledon	1-2
					hypocotyl	0
					shoot tip	1
HC-11		0.5	IAA	1.0	root	0
					axillary bud	-
					cotyledon	1
					hypocotyl	2-3
					shoot tip	1
HC-15 PVP		4.5		0.25	root	1-2
					axillary bud	1
					cotyledon	2-3
					hypocotyl	2-4
					shoot tip	1
HC-20 AC	ZEA	0.5	NAA	0.2	root	1
					axillary bud	1-2
					cotyledon	1-2
					hypocotyl	2-3
					shoot tip	1
HC-22 PVP	BA	0.5	IAA	0.2	root	0
					cotyledon	1-2
					axillary bud	1-3
					hypocotyl	0
					shoot tip	1

Browning of the callus could not be prevented by addition of PVP or activated charcoal. However, addition of activated charcoal (3 mg/l) in the medium delayed the browning of the callus and initially growth of the callus was also good. These supplements had no significant effect on the percentage callus response (Table 17).

4.1.5 Induction of rooting

The summarised results obtained with rooting of the isolated shoots are presented in Table 18. The roots were visible within 25-28 days of incubation on rooting medium. Ten media combinations were tried for inducing roots in the isolated cultured shoots. Rooting was observed in only three media. Highest rooting percentage (60%) was observed on White's medium (WM) + IBA (3.0 mg/l) + KIN (0.04 mg/l). Relatively good rooting (44.4%) was observed when MS basal medium was supplemented with the same combination and concentration of growth regulators. No rooting was observed on medium containing only auxin and no cytokinin.

Thick, light brown coloured roots were produced from the basal end of the stem on the medium containing IBA (3.0 mg/l) and KIN (0.04 mg/l) and thin light brown and long roots without visible root hairs were produced on the medium containing IAA (6.0 mg/l) and KIN (0.05 mg/l) (Fig. 16).

4.1.6 Transfer of plants to soil

Regenerated plants with well developed roots were taken out along with the medium from the culture vessels and rinsed to remove traces of agar, which might provide a substrate for microorganisms. Bavistin (0.5%) was sprayed once a week. Maintaining humidity by covering the plant with a beaker helped to maintain plant under soil conditions. Observations for plant transfer to soil were recorded after 1½ month. Transferring plant to half-strength MS liquid medium

Fig. 16 Differentiation of roots from cut end of regenerated shoot placed on MS + IAA (6.0 mg/l) + KIN (0.05 mg/l).

Fig. 17 In vitro developed plantlet of P. cineraria kept for hardening in half strength MS liquid medium.

Fig. 18 Plantlet transferred to soil : vermiculite mixture.

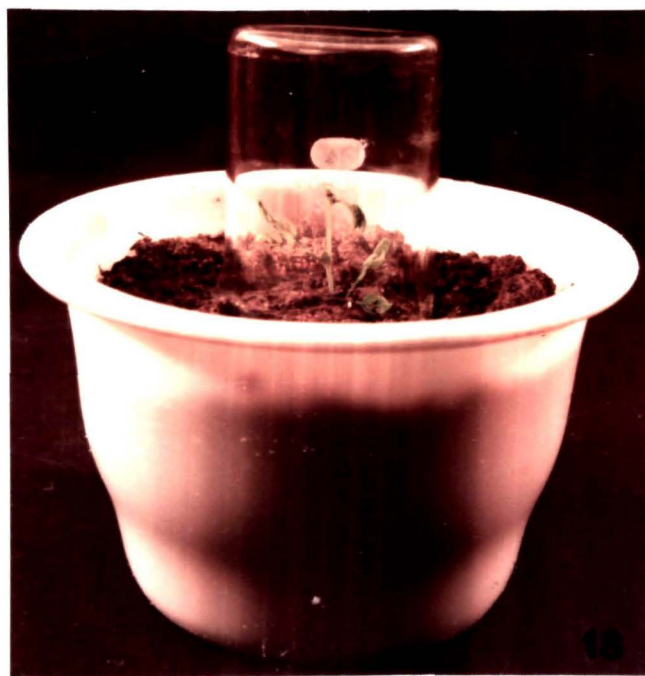


Table 17 Effect of different supplements on callus induction in hypocotyl segments of P. cineraria

Medium : M S + NAA (0.2 mg/l) + ZEA (0.5 mg/l)				
Supplements (mg/l)	Number of explants inoculated	Callus		Colour
		Number	Per cent	
Control	60	57	95.1±0.9	Cream
PVP (360 mg)	40	32	80±0	Yellowish green
Activated charcoal (3000 mg)	40	38	94.5±1.5	White

Table 18 Response of regenerated shoots of P. cineraria in different media for root induction

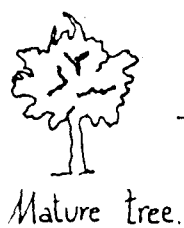
Medium code	Growth regulators				Number of explants cultured	Response %
	Auxin (mg/l)	Cytokinin (mg/l)				
HCR-1	IBA	3.0	-		24	0
HCR-2		3.0	KIN	0.04	54	44.44
HCR-3		3.0		0.05	10	0
HCR-4		4.0	-		15	0
HCR-5		4.0	KIN	0.05	15	0
HCR-6	IAA	0.2	-		15	0
*HCR-7	IAA NAA	2.0 2.0	-		16	0
HCR-8	IAA	6.0	KIN	0.05	24	25.00
HCR-9	NAA	0.5	BA	1.0	9	0
**HCR-10	IBA	3.0	KIN	0.04	15	60.00

* MS half strength

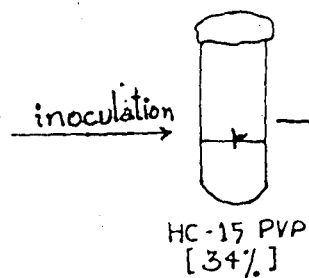
** White's medium

before soil transfer was found to be of no use for successful transfer of plants to soil (Fig. 17). Per cent success was 26.7% (Fig. 18).

On the basis of experimental results, we propose a protocol for in vitro multiplication of Prosopis cineraria from different explants of seedling, juvenile and mature trees and suitable medium for each explant for shoot regeneration, rooting and conditions for plantlet transfer to soil are illustrated in Fig. 19.



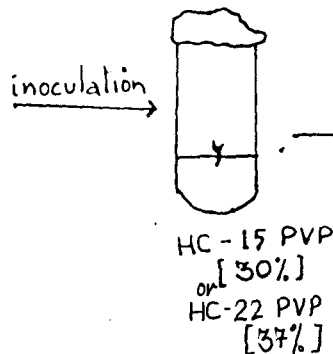
Axillary bud



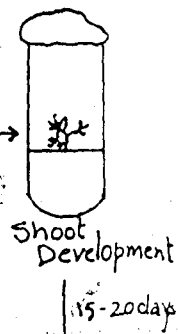
20-25 days



Axillary bud



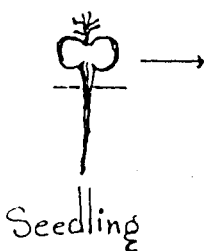
20-25 days



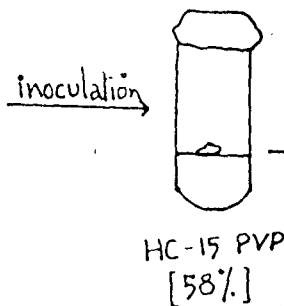
Shoot Development

15-20 days

Rooting
HCR-10
60%
or
HCR-2
44%



Cotyledon



10-15 days

25-30 days

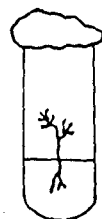


Regenerated Plantlet
transferred from test tube
to soil.



Hardening
 $\frac{1}{2}$ MS Liquid medium

20 days



Regenerated Plantlet.

FIG:19 Protocol for in vitro multiplication of Prosopis cineraria (L) Druce

DISCUSSION

In the present investigation on clonal propagation of Prosopis cineraria (L) Druce an attempt has been made to devise suitable media combinations for plant regeneration from various vegetative explants excised from seedling, juvenile and mature trees. Results obtained in different experiments of the present investigation are discussed in the light of literature available.

In the present study, callus induction was observed in most of the media from hypocotyl, root, cotyledon and internodal and axillary bud (from juvenile and mature tree) explants, however, shoot tip explants of seedling, juvenile as well as mature tree did not produce callus in any of the media under study. Leaves of juvenile and mature tree were also found unresponsive for callus induction. Hypocotyl and root segments were found highly responsive followed by internodal segments and axillary buds. Cotyledonary explants produced callus in three media only viz. HC-10 PVP, HC-15 PVP and HC-22 PVP (Table 7). Goyal and Arya (1981) have

also observed callus formation along with shoot regeneration in hypocotyl segments of P. cineraria. Hypocotyl segments have been found highly responsive for callus formation in many tree species (Durzan and Lopushanski, 1975; Khattar and Mohan Ram, 1983), however, Rao et al. (1988) have found that cotyledonary explants are the best source of callus induction in Azadirachta indica followed by leaves. Role of phytohormones in tissue culture studies has been emphasized by many workers (Chalupa, 1975; Durzan and Lopushanski, 1975; Bhargava et al., 1981; Rao et al., 1988). King and Morehart (1987) reported in Acer rubrum that optimal growth of callus depended on the ratio of cytokinin/auxin as well as the total amount of plant growth regulators in the medium.

When explant response was compared in different media, no single explant produced callus in all the media tried. Similarly no particular medium responded to each and every explant for callus induction. This shows that every explant behaves specifically in each medium under study with respect to per cent callus formation. This may be due to difference in internal hormonal levels of the explant and as well as other physiological factors favouring or inhibiting callus formation. Analysis of variance also confirms this observation as media, explants and their interactions have been found highly significant (Table 13). Callus formation and its regeneration is important and pre-requisite for any genetic manipulation work for tree improvement. Our studies indicate that callus induction and maintenance is possible from different explants discussed above, however, all attempts to regenerate these calli failed during present investigation. This aspect was also not seriously tried as our objective was micropropagation rather than regeneration from callus cultures. Furthermore regeneration from callus cultures is not desirable from micropropagation point of view as it requires absolute fidelity of genotype during in vitro culture.

Different explants exhibited variable response for shoot formation in media under consideration. Among seedling explants, cotyledons showed maximum regeneration potential (58.0%) on HC-15 PVP medium (Table 7). Shoot tip explants gave 50.0% shoot formation on HC-20 AC medium (Table 8). Cotyledonary explants have been found to have higher morphogenetic potential in many trees such as Tamarindus indica (Jaiwal and Gulati, 1991) and Anogeissus pendula (Joshi et al., 1991). Hypocotyl and root segments were relatively low responsive showing maximum response of 20% and 19% respectively under present culture conditions (Table 5 & 6). Regeneration of shoot buds from hypocotyl explants have been reported in Santalum album (Rao and Bapat, 1978) and Albizzia lebbek (Mukhopadhyay et al., 1980) including Prosopis cineraria (Goyal and Arya, 1981). However, Goyal and Arya (1981) have not reported per cent success for shoot formation. Kackar et al. (1992) have observed 7-8 adventitious buds in each root explant of Prosopis cineraria on MS medium containing NOA. In our studies, shoot regeneration frequency was low and this may be due to the fact that we have not used NOA in culture media.

Among juvenile and mature tree explants, shoot regeneration was achieved from axillary buds only and internodal segments, leaves and shoot tip explants completely failed to regenerate shoot buds. It is interesting to note that half of shoot tip explants of seedlings regenerated shoots and same explants from juvenile and mature tree were totally unresponsive under present conditions. This may be due to different physiological state in juvenile and mature tree as compared to seedlings.

Shoot bud formation from internodal explants is reported in Populus deltoides (Coleman and Ernst, 1989) and Dalbergia lanceolaria (Anand and Bir, 1984).

Similarly leaf tissue has been used for plant regeneration in Populus balsamifera (Bychankova and David, 1978) and Dalbergia lanceolaria (Anand and Bir, 1984). Recalcitrant nature of these explants in our studies may be due to genotype specificity or optimal culture conditions were not provided and media used in present study were not favourable for shoot formation.

The shoot regeneration from axillary bud of juvenile tree was highest (37.9%) when the medium contained BA (0.5 mg/l) and IAA (0.2 mg/l) (Table 10). Relatively good regeneration frequency was also observed when the medium contained IAA (0.25 mg/l) and KIN (4.5 mg/l). In mature tree axillary bud, highest regeneration percentage (34) was realised on HC-15 PVP medium (MS + IAA 0.25 mg/l + KIN 4.5 mg/l) (Table 12). Sharma and Chandra (1987) found the combination of BA and IAA better over kinetin combination for shoot bud differentiation from axillary bud explants in Albizia lebbek. Lakshmisita and Shobha Rani (1985) also reported the shoot formation from nodal explants on a medium fortified with BA (0.5 - 2.0 mg/l) and NAA (0.1 - 1.0 mg/l). Datta and Datta (1983) observed the rooted shoots from nodal explants of Dalbergia sissoo when cultured on MS medium with IAA (1 mg/l) and NAA (0.5 mg/l) or NAA only. In Melia azedarach nodal bud segments responded on MS medium supplemented with BA (1.0 mg/l) (Ahmed et al., 1990). All these reports point out that every genotype/ tree species has specific nutritional and hormonal requirement and each genotype has to be standardized to make it amenable for in vitro culture and plant regeneration. Five media namely HC-6 PVP, HC-10 PVP, HC-15 PVP, H-20 AC and HC-22 PVP were found suitable for shoot regeneration response for most of the explants used in study, however, no single medium was found best for all the explants and similarly no single explant responded in all the media under study. Analysis of variance showed that explant, medium composition and their interactions were highly significant for shoot generation at 1% level (Table 13).

Multiple shoot formation from different explants has been reported from nodal explants in Acacia auriculiformis (Mittal et al., 1989) cotyledonary and epicotyledonary nodes in Anogeissus pendula (Joshi et al., 1991). In our studies, different explants showed differential response for multiple shoot formation in different media. Hypocotyl segments showed multiple shoot formation on HC-11, HC-15 PVP and HC-20 AC media (Table 15). Root explants showed multiple shoot formation on HC-10 PVP and HC-15 PVP medium. In axillary bud explants multiple shoot buds were observed on HC-22 PVP and HC-20 AC media. Shoot tip explant did not show any multiple shoot buds. Therefore, it suggests that multiple shoot formation depends upon media composition i.e. hormonal balance, auxin- cytokinin interaction which may or may not be favourable for this process.

During initial culture of explants from juvenile and mature tree, browning is the major bottleneck for dedifferentiation and it makes the cultures hard to maintain and regenerate. Polyphenoloxidases are activated in the tissue and cell metabolism is changed. The phenolic compounds which produce quinonic compounds after oxidization diffuse gradually in the medium and inhibit activities of other enzymes and damage the whole explant which ultimately turns brown. The antioxidants helps to reduce the process of oxidization which leads to browning of explants.

Addition of PVP in the media was not able to prevent the browning of callus. Whereas, activated charcoal delayed the process of browning which indicates that it may soak the phenolic compounds excreted in the medium by the explant. It has been reported in date palm also (Sharma et al., 1988) that activated charcoal reduces browning. However, presoaking of explants for 3 hrs in an antioxidant solution (citric acid 400 mg/100 ml) and then culturing in a medium with PVP prevented complete browning of medium and the explant. Rai and Chandra (1988)

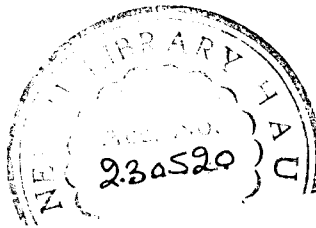
also reported in Dalbergia latifolia that browning of callus and medium could be prevented by adding soluble PVP-10 in the medium. From the above discussion it can be concluded that by soaking in antioxidant solution and then culturing on media with PVP or activated charcoal, the browning of medium/explant can be overcome.

Shoot regeneration is of no practical value without proper rooting in regenerated shoots. Importance of auxins in root formation is well documented (Torrey, 1967) however basal media and auxin (kind and concentrations) vary from species to species (Carswell and Loey, 1984; Profumo et al., 1985). Upadhyay and Chandra (1983) have used liquid MS medium for rooting in Albizzia lebbek. Goyal and Arya (1984) and Arya and Shekhawat (1986) used WM + IBA (3.0 mg/l) + KIN (0.05 mg/l) for rooting in regenerated shoots of Prosopis cineraria. Kackar et al. (1991) found that use of auxins like IAA and IBA (0-2 mg/l) single or in combination resulted in no or poor root initiation in P. cineraria.

In the present investigation White's medium with IBA and kinetin (HCR-10) gave highest rooting percentage (60%). HCR-2 with MS basal medium also gave relatively good response (44.4%) for adventitious root formation (Table 18).

Different types of substrates have been used under the acclimatization period before transfer of plantlets to soil. Soil-vermiculite mixture (Goyal and Arya, 1981, 1984), sterilized sand (Bhansali et al., 1988), soil (Kurten et al., 1990). Durand-Cresswell et al. (1982) transferred in vitro raised eucalypts plantlets to "melfert balls". For Eucalyptus marginata, McComb and Bennett (1982) used a mixture of two parts sand to one part sphagnum peat. Acacia koa plantlets were allowed to develop normal well-branched roots with root hairs by growing in Hoagland's solution before transplanting to potting mix (Skolmen and Mapes, 1978).

In the present investigation agar was removed from regenerated shoots and then the plantlets were transferred to pot containing a mixture of soil : vermiculite (3:1 v/v) with 26.7% success. Some of the plantlets were transferred to half strength MS liquid medium/water before transplanting to pot. However, this did not help in successful transfer of plants to soil.



SUMMARY

During the present investigation attempts have been made to develop protocols for in vitro multiplication of Prosopis cineraria (L) Druce. The salient features of the results achieved are as follows:

1. Among seedling explants, except shoot tip all the explants responded for callus induction. Hypocotyl and root explants were the best for callus induction. In both of these explants maximum callusing was recorded on MS medium containing NAA (0.2 mg/l) and ZEA (0.5 mg/l). In cotyledonary explants, callusing was observed in only three media and maximum percentage of callusing was observed on HC-22 PVP medium containing IAA (0.2 mg/l) and BA (0.5 mg/l).
2. In case of juvenile tree explants, callus could be induced in internodal segment and axillary bud. For internodal explants maximum percentage of callusing (62.4%) was obtained on HC-22 PVP medium containing IAA (0.2 mg/l) and BA (0.5 mg/l), although it did not differ significantly from HC-15 PVP and

HC-17 AC medium. In case of axillary buds, maximum callus induction was observed on HC-17 AC medium with IAA (2.0 mg/l) and KIN (6.0 mg/l). There was no or poor callus response on medium containing high auxin and low Kinetin.

3. Among mature tree explants, callus formation was obtained in internodal and axillary bud explants. In both the explants, percentage callus induction was highest on HC-22 PVP medium containing IAA (0.2 mg/l) and BA (0.5 mg/l). Low Kinetin and high auxin in the medium resulted in no or low response for callus induction.

4. Among the seedling explants, viz. hypocotyl, root, cotyledon and shoot-tip, used for plant regeneration, the cotyledon explants showed considerably higher shoot regeneration potential than the others. For seedling explants high regeneration frequency was realised on HC-15 PVP medium containing low IAA (0.25 mg/l) and high Kinetin (4.5 mg/l).

5. Among juvenile and mature tree explants, regeneration could be achieved only from axillary bud and the highest regeneration frequency in juvenile as well as mature tree was obtained on HC-22 PVP and HC-15 PVP media respectively.

6. Young leaves and shoot tip explants from juvenile and mature tree remained unresponsive for callus induction as well as shoot regeneration in media under study.

7. The best callus induction response was observed in hypocotyl segments followed by internodal explants from juvenile and mature tree, while shoot regeneration was the best in cotyledonary explants followed by seedling shoot tip explants and axillary buds from juvenile and mature tree under culture conditions of present investigation.

8. Multiple shoot formation was found to be dependent on media-explant interaction. No single medium was found to induce multiple shoot formation in all the explants as well no explant exhibited this phenomenon on all the media

under study. In general, cotyledonary and hypocotyl explants developed multiple shoots on HC-15 PVP and HC-20 AC media. Root segment produced multiple shoots on HC-10 PVP and HC-15 PVP media. Juvenile as well as mature tree axillary buds developed multiple shoots on HC-22 PVP and HC-20 AC media.

9. Browning in callus cultures was observed during the present investigation. Addition of PVP (360 mg/l) had no effect in preventing browning of callus. Addition of activated charcoal (3 g/l) delayed it rather than prevented it. However, presoaking of explants from juvenile and mature tree, in antioxidant solution for three hours and thereafter culture on PVP (360 mg/l) containing media completely removed browning of medium and explant.

10. Media composition, source of explants as well as their interaction was found highly significant for callus induction and shoot regeneration in the present study.

11. Rooting in regenerated shoots was found best in White's medium containing IBA (3.0 mg/l) and Kinetin (0.04 mg/l). The complete plantlets were transferred to small pots containing a sterile soil : vermiculite mixture with 26.7% success.

12. Procedures for the development of complete plants through plant tissue culture method have been identified from different vegetative explants of P. cineraria.

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* Original not seen

