ENGINEERING ANTIOXIDANT DEFENSE MECHANISM TO COMBAT SALINITY STRESS IN *Brassica juncea*

Thesis

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By

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On the verge of thesis submission I am overwhelmed with the mixed feelings coming to my heart. It is difficult to transform feelings exactly to words; still it is an attempt to give tribute to those who made this task possible.

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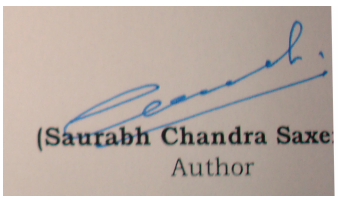

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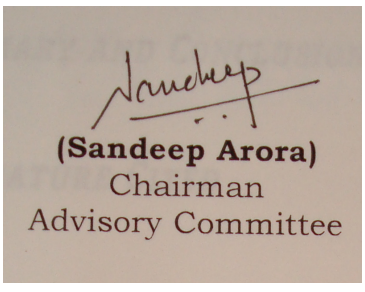
CERTIFICATE

This is to certify that the thesis entitled **"ENGINEERING ANTIOXIDANT DEFENSE MECHANISM TO COMBAT SALINITY STRESS IN *Brassica juncea*"** submitted in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** with major in **Biochemistry** and minor in **Environmental Sciences** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona-fide* research carried out by **Mr. Saurabh Chandra Saxena, Id. No. 29636**, under my supervision and no part of the thesis has been submitted for any other degree or diploma.

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



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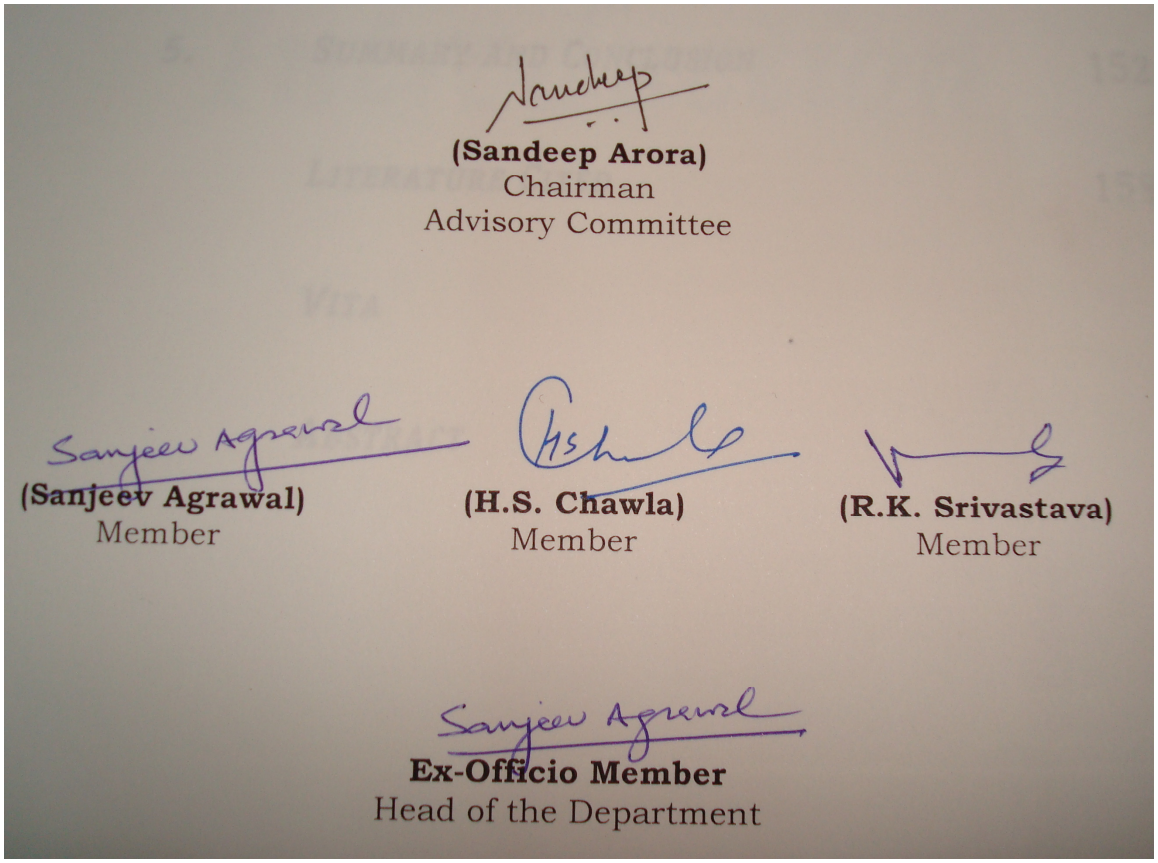
We, the undersigned, members of the Advisory Committee of **Mr. Saurabh Chandra Saxena, Id. No. 29636**, a candidate for the degree of **Doctor of Philosophy** with major in **Biochemistry** and minor in **Environmental Sciences**, agree that the thesis entitled **"ENGINEERING ANTIOXIDANT DEFENSE MECHANISM TO COMBAT SALINITY STRESS IN *Brassica juncea*"** may be submitted in partial fulfilment of the requirements for the degree.



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Introduction

Chapter 1 INTRODUCTION

World market prices for major food commodities such as grains and oilseeds have risen sharply to historic highs of more than 60 percent above levels observed just 2 years ago. Such unprecedented and sharp increase in food prices has sparked riots in many parts of the world in 2008 and has compelled at least 30 governments to impose emergency measures such as food price controls or export restrictions on specific food items. Important agricultural commodity prices continued the rising trend into early 2008, competing with oil-price hikes in capturing economic planner's and policy maker's attention. Thus, world food security and agriculture are facing critical challenges in the present decade. These developments have led to a growing awareness and a justifiably heightened concern about food and nutritional security, especially in developing countries, where food availability at affordable prices is precarious.

India is basically an agrarian economy. Agriculture in India accounts for nearly 65% of the country's employment, 20% of the Gross Domestic Product and nearly 20% of total export earnings, annually. Agriculture is not only the backbone of Indian economy and food security but is also a way of life, a tradition and an anchor of overall livelihood opportunity for about 700 million of our one billion population. Agriculture, therefore, is and will continue to be central to all strategies for planned socio-economic development of the country.

As per census of India 2001, India's total population stood at 1,028.6 million and the percentage of decadal growth in population during 1991-2001 was 21.34 per cent. Thus, one of the main challenges, for agricultural scientists is to ensure enough food for this burgeoning population. Though the food grain production in recent

years has reached record levels, still India has to produce an additional 5-6 million tons of food grains annually, in the next couple of years to meet the requirements of an estimated population of nearly 1,230 million people by 2010 **(Handbook of Agriculture, 2006a)**.

In the Indian agricultural economy, oilseed crops occupy a unique position, next only to food-grains in terms of total hectarage, production and economic value. *Brassica* (rapeseed-mustard) is the second most important edible oilseed crop in India, after groundnut and accounts for nearly 25-30% of the total oilseeds produced in the country **(Handbook of Agriculture, 2006b).**

In the International arena India is a major rapeseed-mustard growing country of the world, standing at second position in terms of total production after China and first position in terms of area cultivated. India contributes 28.3 and 19.8 per cent in world acreage and production, respectively **(FAO, 2008a)**. The productivity of rapeseed-mustard has increased in India from 870 kg/hectare in 1998-99 to 1106 kg/hectare in 2001-02. This marked increase in the productivity of rapeseed-mustard, visible in all rapeseed-mustard growing States as well as in non-traditional areas of the country, has rightly been termed as *Yellow Revolution*.

In spite of these achievements, there exists a considerable gap between production potential and actual realization. The projected demand for oilseed crops in India is around 34 million tones by 2020, of which about 14 million tones (41%) is to be met by rapeseed-mustard. Productivity of rapeseed mustard in India varies significantly from State to State, depending on the climatic conditions and other environmental factors prevailing in the respective areas. The current range of productivity varies from 800 to 1235 Kg/ha. A number of socio-economic as well as environmental factors influence the production potential & the actual production realization and the

consequent run-up in food commodity prices. Recent factors that have further tightened Indian markets include increased demand for bio-fuels, feed-stocks and adverse weather conditions in 2006 and 2007 in some major grain and oilseed producing areas. Among the various environmental factors, abiotic stresses constitute one of the most important factors influencing the overall productivity of crop plants.

For a secure and progressive Indian economy, revitalized agriculture can serve as the engine of growth. To address 'head-on' the challenge of feeding its increasing population with its limited economic, land and water resources, there is an urgent need to improve the productivity of major cash crops by bringing under cultivation the hitherto unutilized or underutilized land areas which are affected by various environmental vagaries and also by harnessing the potential of *Genetic Engineering* for developing improved varieties, by introducing novel stress tolerance traits.

Crop plants, including rapeseed-mustard, are affected by various biotic & abiotic stresses like salinity, drought, low and high temperature, frost damage etc., which translate to significant reduction in yield as well as quality of the produce. Among the various abiotic stresses, salinity and drought are the most widespread ones.

Soil salinity is a major abiotic factor that adversely affects crop productivity. It has been reported that about one-third of world-irrigated land is affected by salinity. In India salinity is a serious yield limiting factor in many parts of the country. About 7 million hectares of land, constituting approximately 17.5% of the total arable land in the country, is affected by salinity **(Singh, 2003)**. In the agricultural scenario the problem of salinity is becoming more and more severe with time, as the non-saline soils and non-saline water resources are being increasingly exploited to accommodate the growing demands of the burgeoning population.

Salinity is detrimental to plants as it causes nutritional constraints by altering the uptake of nitrogen, phosphorus, potassium & calcium and interferes with the cellular metabolism by causing ion toxicity and osmotic imbalances. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the function of these proteins. Ion toxicity, osmotic stress and nutritional defects, under salinity, lead to metabolic perturbations and oxidative stress **(Hirt and Shinozaki, 2004)**. An uncontrolled disturbance in cellular redox homeostasis can cause oxidative stress through the generation of reactive oxygen species (ROS) **(Asada, 1994)**. Reactive oxygen species, such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) etc., which are highly reactive, may cause cellular damage through oxidation of lipids, proteins and nucleic acids **(Pastori and Foyer, 2002; Apel and Hirt, 2004).**

Incidentally, reactive oxygen species are produced in both unstressed and stressed conditions, and at various intracellular locations **(Halliwell and Gutteridge, 1989; Rizhsky *et al.*, 2004).** However, uncontrolled production of ROS during exposure to environmental stresses, beyond the inherent detoxifying capacity of the cells, is one of the main causes for decrease in plant growth and development.

During the course of evolution plants have developed several strategies to defend themselves against reactive oxygen species generated under environmental stresses **(Foyer *et al.*, 1994; Inze and Van Montagu, 1995)**. The antioxidant defense system of plants includes, antioxidant molecules such as ascorbate, glutathione, carotenes and α–tocopherol; which directly play an important role in the detoxification of ROS **(Alscher *et al.*, 1997)** and antioxidant enzymes like ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and Monodehydro ascorbate

reductase (MDHAR) **(Foyer *et al.*, 1994; Allen *et al.*, 1995)**; which show an increased activity as a result of exposure to stress.

However, in spite of having a potential antioxidant defense system, plants do suffer extensive damage on exposure to stressful conditions. This indicates that the plant's defense system has a limited capacity to detoxify reactive oxygen species produced as a result of stress. It has also been observed that the strength of antioxidant defense system varies from species to species. Some species have a potent antioxidant defense system and can withstand exposure to a higher degree of stress, as compared to other species. Therefore, strengthening the antioxidative defense of important crop plants might help them to overcome the deleterious effects of reactive oxygen species and survive successfully under stressful conditions.

Out of various reactive oxygen species, hydrogen peroxide (H_2O_2) is one of the most toxic forms of reactive oxygen species. Incidentally, at lower concentrations H_2O_2 acts as a signal transducer and serves as a secondary messenger during plant defense. However, exposure to stress exacerbates H_2O_2 production in the cells, beyond the critical limits and converts it into a toxic molecule. Since H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, its accumulation beyond a critical level causes extensive cellular damage.

Hydrogen peroxide is relatively stable and is electrically neutral; therefore, it can penetrate through biological membranes with relative ease, thereby increasing its scope of cellular damage. In the presence of ferrous iron (Fe^{2+}), or other ions capable of a single electron redox change (e.g. Cu^{2+}), it can be further reduced by superoxide radicals *via* the Haber-Weiss reaction to form highly reactive and cytotoxic hydroxyl radical ($HO\cdot$), thereby leading to a cascading effect.

Excess H_2O_2 in a cell is potentially taken care of by catalase and peroxidases. Ascorbate peroxidase is one such enzyme that helps

regulate the levels of H_2O_2 in plant cells. Ascorbate peroxidase is an integral part of H_2O_2 regulating pathway (ascorbate-glutathione cycle), and is also one of the most widely distributed antioxidant-enzyme in plant cells **(Wang *et al.*, 1999)**. Ascorbate peroixdase in combination with an effective AsA-GSH cycle, functions to prevent the accumulation of toxic levels of H_2O_2 in almost all higher plants **(Asada, 1992, 1997).** Reactive oxygen species scavenging enzymes are present in different cellular compartments as specific isoenzymes. Ascorbate peroxidase also exists in several isozymic forms and plays a critical role in the metabolism of H_2O_2 in higher plants **(Alscher *et al.*, 2002; Shigeoka *et al.*, 2002)**. Among the different APX isoenzymes, cytosolic form is reported to be the most responsive to various environmental stresses **(Yoshimura *et al.*, 2000).**

Manipulation of the expression of enzymes involved in the ROS scavenging systems by way of gene transfer technology has provided a powerful tool for increasing the present understanding of the potential of defense network against oxidative damage caused by environmental stresses, apart from strengthening its antioxidative defense system. We hypothesize that enhancing the activity of ascorbate peroxidase enzyme, through genetic engineering; will strengthen the antioxidative defense system and will enable it to limit the increased production of hydrogen peroxide under stress. For the present investigations we have chosen *Brassica juncea* as the target crop, which is one of the important oilseed cash crop in India and whose productivity is significantly affected by salinity stress.

In the light of above discussion the present investigations were carried out with the following objectives:

1. Standardization of an efficient and reproducible regeneration and transformation protocol for *Brassica juncea* variety pusa jaikisan.

2. Over-expression of cytosolic ascorbate peroxidase gene (*apx1)* in *Brassica juncea* through genetic transformation.
3. Biochemical and molecular analysis of the putative transgenic *Brassica juncea* plants, to study the expression and activity of ascorbate peroxidase.
4. Evaluating the antioxidant response of transgenic *Brassica juncea* plants towards salinity stress.

Review of Literature

Chapter 1

REVIEW OF LITERATURE

The cultivation of plants was one of the greatest revolutionary accomplishment that presumably began in the Mesolithic or Middle stone age from 12000 to 6000 BC, when man lived with spear, bow and fishing net. Since then the increasing needs urged him to search for better techniques to fulfill his requirements. In the present 'Space age', agriculture faces many challenges worldwide due to interaction of several phenomenon, important being 'population explosion' and changing environmental conditions.

The world human population is expected to reach 8.0 billion by 2025 and 8.9 billion by 2050. About 80 million people are being added to the population each year, and 97% of the predicted population growth will take place in the developing countries. It is projected that there is a need to double world food production in order to feed 8.0 billion people by 2025 **(FAO, 2008)**. This will certainly place more pressure on the environment. The developing countries, in particular, are confronted with severe food-security challenges. With a severe limit to the amount of unused land available to bring into cultivation, improving crop yields in both normal soils and less productive lands, including salt-affected lands, is an absolute utter requirement to satisfy future world food needs.

Agriculture is the largest private enterprise in India, has been and will continue to be the lifeline of the Indian economy at least in the foreseeable future. It contributes nearly 20% to the national GDP, sustain livelihood of about two third of the population, provide direct employment to about 234 million people and forms the backbone of the agro-based industry.

India's greatest achievement in the past century has been its ability to increase its food production and thus keeping the Malthusian fears at bay. Nevertheless, we still face daunting challenge of hunger, poverty and malnutrition that will only get worse as the population increases in absolute numbers. Recent advances in crop science, however, provide us with a new window of opportunity to deal with the issue of food security.

Agricultural commodity markets are expected to remain tight in the future, and prices are expected to remain higher in the coming decade than they were in the past decade. Agricultural prices have always been volatile, but recent sharp increases in global agricultural commodity prices have focused unprecedented attention on the state of food and agriculture at the global, regional and national levels **(OECD–FAO, 2008)**.

Indian vegetable oil economy is the fourth largest in the world, next to US, China and Brazil, accounting for about 13 per cent of the oilseeds area, 7 per cent of the world's oilseeds production and 10 per cent of world's edible oil consumption. India is amongst the largest producer and consumer of vegetable oils **(Handbook of Agriculture, 2006b).**

In India, rapeseed-mustard are the second largest oilseed crops after the groundnut and grown mainly in north-western and central part of India in different ecosystems and cropping sequences. *Brassica juncea* is a promising oilseed crop in India growing under a wide range of climatic conditions with low inputs on marginal and sub marginal lands during rabi season (October - March). Indian mustard (*Brassica juncea*) is cultivated in the states of Assam, Bihar, Gujarat, Haryana, Himachal Pradesh, Jammu & Kashmir, Madhya Pradesh, Orissa, Punjab, Rajasthan, Uttar Pradesh, West Bengal and Uttarakhand.

2.1 *Brassica juncea*

Under the name rapeseed mustard, seven important annual oilseeds belonging to the Brassicaceae (Cruciferae) are grown in India. They are Indian mustard (*Brassica juncea* [L.] Czern. & Coss.), commonly called rai (raya or laha), the three ecotypes of Indian rape, *Brassica campestris* L. ssp. oleifera viz., toria, brown sarson (lotni and tora types) and yellow sarson, Swede rape or gobhi sarson (*Brassica napus* L.), Ethiopian mustard or karan rai (*Brassica carinata* Braun.) and taramira or tara (*Eruca sativa* Mill.).

In the Indian subcontinent *B. juncea* is the dominant species grown and along with. *B. rapa* (syn. *B. campestris* L.) and *B. napus* L. are the important sources of edible oil in India. These species are regarded as of Asiatic origin. Variation in Varieties along with local names and chief characteristics are shown in table 1.

Table 1: Chief characteristics and common names of *Brassica* species

Sl. No.	Botanical Name	English name	Common Name	Chief Characteristics
1.	*Brassica campestris*	Rapeseed	Sarson	The seeds are bold or large size, round in shape and have a smooth surface, colour of the seed yellow or brown or dark brown to black.
2.	*Brassica campestris*	Rapeseed	Toria	Seeds are spherical or avoid in shape and are reddish or dark brown in colour, having slightly wrinkled surface. Seeds are slightly smaller than those of Sarson.
3.	*Brassica juncea*	Mustard	Rai	Seeds are small in size spherical or ovoid in shape and have a distinctly wrinkled surface. Colour of the seed is dark brown or black.

2.1.1 History

Brassica oilseed crops (also referred as rapeseed-mustard) comprise of *B. juncea, B. rapa, B. napus, B. carinata* are believed to be originated from central Asian-Himalayas with migration to India, China, Middle east, Europe and Northeastern Africa, respectively. *Brassica juncea* is a tetraploid - it has double the number of chromosomes normal for the genus. It apparently originated in Asia by hybridization between *B. rapa* (a variable species which includes turnip, Chinese, cabbage, pak choi, and broccoli raab) and *B. nigra* (black mustard, the species generally grown for the seeds from which the yellow condiment is made). (Fig. 1)

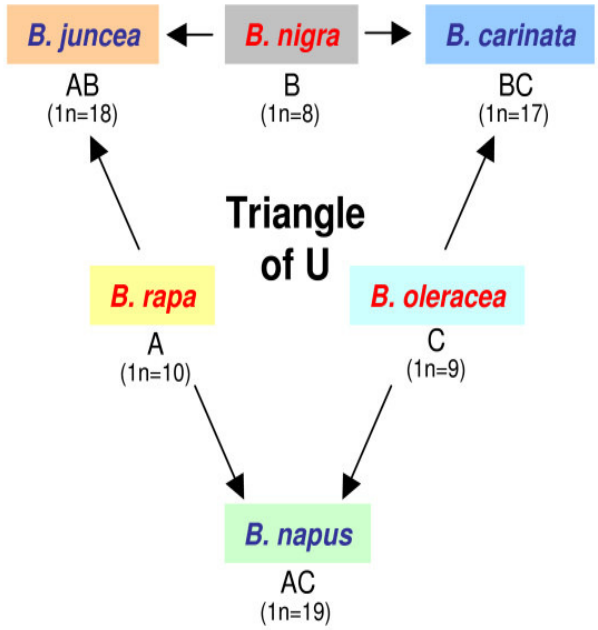


Fig.1: The genetic relationship between *Brassica* species of the "Triangle of U" (U.N., 1935).

2.1.2 Description

Perennial herb, usually grown as an annual or biennial, upto 1 m or more tall; branches long, erect or patent; lower leaves petioled, green, sometimes with a whitish bloom, ovate to obovate, variously lobed with toothed, scalloped or frilled edges, lyrate-pinnatisect, with

1–2 lobes or leaflets on each side and a larger sparsely setose, terminal lobe; upper leaves subentire, short petioled, 30–60 mm long, 2–3.5 mm wide, constricted at intervals, sessile, attenuate into a tapering, seedless, short beak 5–10 mm long (fig.2). Rooting depth is 90–120 cm **(Duke *et al.*, 1981).**

Fig.2: A descriptive photo of *Brassica juncea*, showing different floral parts

2.1.3 Environmental Requirements:

A. Climate

Mustard is a cool season crop that can be grown in a short growing season. Varieties of yellow mustard usually mature in 80 to 85 days whereas brown and oriental types require 90 to 95 days. Seedlings are usually somewhat tolerant to mild frosts after emergence, but severe frosts can destroy the crop. Mustard, especially the brown and oriental types, has a partial drought tolerance between that of wheat and rapeseed.

B. Soil

Mustard can be raised on variable soil types with good drainage, but is best adapted to fertile, well-drained, loamy soils. Soils prone to crusting prior to seedling emergence can cause problems. This crop will not tolerate waterlogged soils since growth will be stunted. Dry sand and dry sandy loam soils should also be avoided.

C. Seed Germination

Seed will germinate at a soil temperature as low as 40^{o}F.

2.1.4 Cultivation

Seeds are sown in very early spring for spring use and in the fall for winter use. Successive plantings 10–14 days apart insure an all season crop. Sown in drills 30–45 cm apart; plants thinned to about 15 cm as they become crowded in the row. Control of weeds is essential, and 1 to 3 inter-cultivations may be necessary. When grown for seed, off type plants should be rouged before flowering. In India, for pure culture, seeding is at a rate of 4–6 kg/ha **(Knowles *et al.*, 1981)**.

2.1.5 Harvesting

Growing period is from 40–60 days, depending on variety and weather conditions. Plants are generally harvested before fruits fully ripe to reduce the shattering. Harvesting is done usually in early morning. Entire plants are either pulled out by hand or cut a few centimeters above ground with sickles. Plants are tied into small sheaves and dried in the sun for 4–10 days. Extraction of oil from the seed is by rotary mill, expeller and hydraulic processes **(Knowles *et al.*, 1981)**.

2.1.6 Usage of Rape/Mustard seed

Young tender leaves of mustard greens are used in salads or mixed with other salad greens. Older leaves with stems may be eaten

fresh, canned or frozen, for potherbs, and to a limited extent in salads. Mustard greens are often cooked with ham or salt pork, and may be used in soups and stews. Although widely and extensively grown as a vegetable, it is being grown more for its seeds which yield an essential oil and condiment. Easier to grow than Black Mustard (*B. nigra*), it has nearly replaced it in brown mustard preparations since 1945. Mustard Oil is one of the major edible oils in India. Rai seeds yield fixed oil content varying between 28.6% and 45.7%. Oil is also used as hair oil, lubricants and in Russia, as a substitute for olive oil. Adding 1.1–2.2% mustard oil to fresh apple cider retards fermentation. Seed residue is used as cattle feed and in fertilizers **(Reed, 1976).**

2.1.7 Properties

Brown mustard leaves contain per 100 g edible portion: water 90.8 g, energy 109 kJ (26 kcal), protein 2.7 g, fat 0.2 g, carbohydrate 4.9 g, total dietary fiber 3.3 g, Ca 103 mg, Mg 32 mg, P 43 mg, Fe 1.46 mg, Zn 0.2 mg, vitamin A 10,500 IU, thiamin 0.08 mg, riboflavin 0.11 mg, niacin 0.80 mg, folate 187 µg , ascorbic acid 70 mg. Dry mustard seed contains per 100 g edible portion: water 6.9 g, energy 1964 kJ (469 kcal), protein 24.9 g, fat 28.8 g, carbohydrate 34.9 g, Ca 521 mg, Mg 298 mg, P 841 mg, Fe 10.0 mg, vitamin A 62 IU, thiamin 0.54 mg, riboflavin 0.38 mg, niacin 7.9 mg, ascorbic acid 3 mg **(USDA, 2002).**

The projected demand for oilseeds in India is around 34 million tons by 2020, of which about 14 million tons (41%) is to be met by rapeseed-mustard. Nevertheless, area, production and yield (productivity) of oilseeds in India have been fluctuating because of several biotic and abiotic stresses affecting the crops. Currently, India is importing edible oils to meet internal demands. Dryland rainfed farming is the dominant sector in Indian Agriculture. Over 96.5

million hectare or 68% of net sown area falls under rainfed dryland agriculture. The major crops of the drylands are coarse grains, pulses and oilseeds. About 80% of the area under oilseeds is rainfed (46.2% of the area under rapeseed-mustard is rainfed) **(Statistical Data source CMIE, 2008)**.

2.2 Environmental stress

When any environmental factor interferes with the complete expression of genotype potential, it is called stress. Stress may be said to occur when environmental conditions lead to reduced growth, reduced yield and initiate processes of physiological acclimatization. Environmental stresses can be of two types:

1. Biotic stresses, which mainly belong to the field of pathology and ecology.
2. Abiotic/physicochemical stresses which may occur due to temperature, water (both, excess and deficit), radiations and chemicals, mainly salts and ions **(Levitt, 1980)**.

Environmental stresses represent a major constraint to meet the world food demand. There are relatively few "stress free" areas where crops may approach their potential yields. Exposure of plants to abiotic stresses such as high salinity, drought, extreme light and temperature leads to major loss in crop productivity worldwide.

Abiotic environmental factors (abiotic stresses) are considered to be the main source (71%) of yield reductions **(Boyer, 1982)**. The estimation of potential yield losses by individual abiotic stresses are estimated at 17% by drought, 20% by salinity, 40 % by high temperature , 15% by low temperature , and 8% by other factors **(Ashraf and Harris, 2005)**.

2.2.1 Salinity stress

Among the various abiotic stress factors, soil salinization is the biggest threat to inland agriculture. A study on global land use pattern reveals that 7% of the world's land area, amounting to 1000 million hectares, has become saline **(Tester and Davenport, 2003)**.

In the present scenario, salinity caused by human interference, through use of poor-quality irrigation systems, is a major concern for scientists around the world. Therefore, apart from the need for proper irrigation practices, a concerted effort to understand the effects of salinity on plants, development of genetically engineered crop varieties and superior salt-tolerant cultivars are essential to combat the world's salinization problems **(Rengasamy, 2006)**.

Saline soil is characterized by toxic levels of chlorides and sulfates of sodium. The electrical conductivity (EC) of saturation extracts of saline soil is > 4mmhos cm^{-1}, exchangeable Na percentage is less than 15 and pH is less than 8.5 **(Marschner, 1995).** Salinity is detrimental to plants as it causes various kinds of alterations such as (i) nutritional constraints by decreasing uptake of phosphorus (P), potassium (K), nitrogen (N) and calcium (Ca) (ii) ion toxicity mainly due to Na^+, Cl^- and SO_4^{--} and (iii) osmotic stress: Na^+ competes with K^+ in biochemical reactions, which is inimical to cellular processes. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the non-covalent interactions between amino acids of proteins. This leads to conformational changes and loss of function of proteins. In addition, ion toxicity, osmotic stress and nutritional defects under salinity may lead to metabolic imbalances causing oxidative stress **(Zhu, 2001).**

In arid and semi-arid areas of India, crop production is limited because of soil-salinity and/or-alkalinity. It has been estimated that about 7-12 million hectares of land in the country have either gone

out of cultivation or this area produces low yields of crops **(Agarwal, 1979).**

2.2.2 Non-saline alkali or sodic soils

These soils do not contain any large amount of neutral salts and, as such, the electrical conductivity is < 4mmhos cm^{-1}. The detrimental effect of alkali soil on plants is largely due to toxicity of high amount of exchangeable sodium and the pH. Alkali soils have an exchangeable sodium percentage of more than 15 and pH greater than 8.5. Such soils have low infiltration rate and the physical condition is unfavorable. Because of high alkalinity, resulting from sodium carbonate, the surface soil is discoloured and black, and hence the term black alkali is frequently used to designate the non-saline alkali soil.

2.2.3 Saline alkali soils

This group of soils is both saline and alkali. They have appreciable amounts of soluble salts, as indicated by the values of electrical conductivity which are >4mmhos cm^{-1}. Also, the exchangeable sodium percentage is greater than 15. The pH, however, is likely to be less than 8.5.

2.2.4 Causes of salinity

During the periods of higher than average rainfall, the soluble salts are leached from the more permeable high-lying area to the low-lying areas. This results in the accumulation of salts if the drainage is poor. Moreover, the excessive irrigation of the uplands with water containing salts also results in the accumulation of salts. In areas having a salt layer at lower depth in the profile, seasonal irrigation may favor the upward movement of the salts. Rise in the water table within 2m of the surface due to irrigation, the obstruction of natural

drainage may also cause soil salinity because of developmental activities, e.g. roads and canals and the situation of natural drainage. In the coastal areas, the ingress of seawater induces salinity in the soil.

2.2.5. Effects of salinity stress

About 20% of irrigated agricultural land is adversely affected by salinity **(Flowers and Yeo, 1995).** NaCl stress is a major factor, which limit the crop production because it affects almost all plant functions **(Bohnert and Jensen, 1996).**

Processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by high salt concentration ultimately causing diminished economic yield and also the quality of production. The response depends on the severity and duration of the stress, the developmental stage of the affected plant, the tissue type, and the interactions of multiple stresses. Salt-stress and dehydration stress show a high degree of similarity with respect to physiological, biochemical, molecular and genetical effects. This is possibly due to the fact that sub-lethal salt-stress condition is ultimately an osmotic effect, which is apparently similar to that brought in by water deficit.

Adverse effects of salinity on plant growth may be due to ion cytotoxicity, (mainly due to Na^+, Cl^-, and SO_4^{2-}), and osmotic stress (**Zhu, 2002**). Most crop plants are susceptible to salinity even when ECe is < 3.0 dS m^{-1}. At these salinity levels, the predominant cause of crop susceptibility appears to be ion toxicity rather than osmotic stress. Ion cytotoxicity is caused by replacement of K^+ by Na^+ in biochemical reactions and conformational changes and loss of function of proteins as K^+ and Cl^- ions penetrate the hydration shells

and interfere with noncovalent interactions between their amino acids. Metabolic imbalances caused by ionic toxicity, osmotic stress, and nutritional deficiency under salinity may also lead to oxidative stress **(Zhu, 2002).** Salinity tolerance has been studied in relation to regulatory mechanisms of osmotic and ionic homeostasis **(Ashraf and Harris, 2004).**

2.2.6 Salinity and osmotic stress

In salt-stress affected plants, the result is primarily an ionic imbalance and hyperosmotic stress. The effect of this imbalance or disruption in homeostasis occurs at the cellular level as well as at the whole-plant level (Fig. 3). Massive changes in ionic and water balance cause molecular damage and growth arrest. Finally, in extreme saline conditions, this leads to tissue death and ultimately death of the plant **(Zhu *et al.*, 1997; Xiong and Zhu, 2002)**.

It is evident from the growing body of experimental evidence that salt stress affects the integrity of cellular membranes, activities of enzymes and the functioning of the plant photosynthetic apparatus **(Serrano *et al.*, 1999)**. An important cause of this damage is production of reactive oxygen species (ROS) **(Smirnoff, 1993)**. The most likely sequence of events with increased salinity is: physiological water deficit–abscisic acid regulated stomatal closure in leaves–limited CO_2 availability–over-reduction of electron transport chain, and finally generation of ROS. This condition, termed photooxidative stress, is also the underlying theme in other plant stress responses like the responses to drought, temperature and light stress (**Tanaka et al., 1999; Jiang and Zhang, 2002)**.

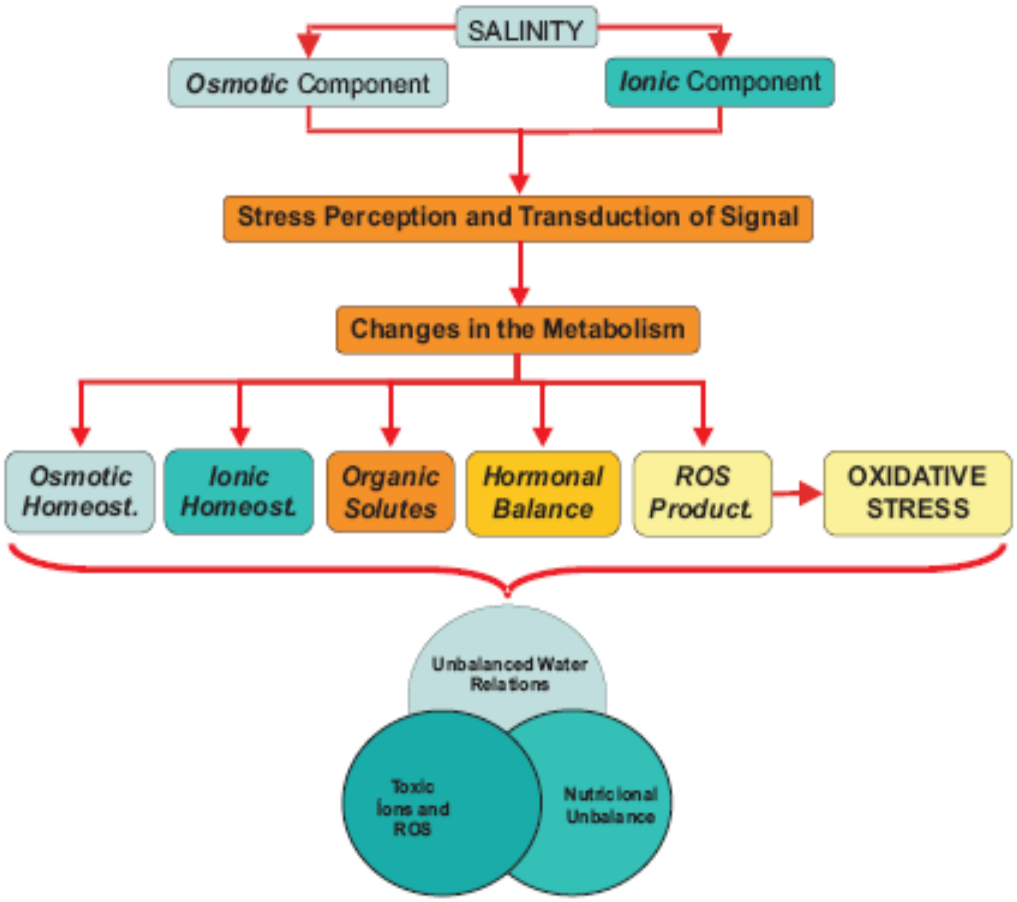


Fig.3: Physiological and biochemical changes occurring in plants when subjected to salt stress

2.3 Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) include free radicals such as superoxide ($\cdot O_2^-$) and hydroxyl radicals ($OH\cdot$) and non free radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are produced by addition of one, two or three electrons to molecular oxygen to form $\cdot O_2^-$, H_2O_2 and $OH\cdot$ respectively or by photosensitized chlorophyll yielding $^1O_2$ under normal conditions **(Mittler, 2002)**. They are also produced in response to both biotic and abiotic stresses such as drought, exposure to UV light, ozone, extremes in temperatures, elevated light intensity and pathogen challenge **(Bartosz, 1997)**.

ROS are produced in both unstressed and stressed cells and in various locations **(Halliwell and Gutteridge, 1989)**. ROS are capable of damaging any biomolecules including proteins, sugars, fatty acids and nucleic acids.

Production of ROS during environmental stress is one of the main causes for decreases in productivity, injury, and death that accompany these stresses in plants. but they play a central role in many signaling pathways in plants involved in stress perception, photosynthesis regulation, pathogen response, programmed cell death, hormonal action, and plant growth and development **(Dat *et al.*, 2000; Mittler, 2002; Mullineaux and Karpinski, 2002; Apel and Hirt, 2004)**.

2.3.1 Chemistry of ROS

To oxidize a non-radical atom or molecule, dioxygen would need to react with a chemical species that provides a pair of electrons with parallel spins that fit into its free electron orbitals. Fortunately, pairs of electrons typically have opposite spins, and thus, impose a restriction on the reaction of molecular oxygen with most organic molecules, such as amino acids and nucleic acids. Dioxygen, however, may be converted to ROS either by energy transfer or by monovalent reduction (Fig. 4). If oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet oxygen ($^1O_2$), in which the two electrons have opposite spins. Since paired electrons are common in organic molecules, singlet oxygen is much more reactive toward organic molecules than dioxygen in its ground state. The second mechanism of activation of oxygen is by the stepwise monovalent reduction through electron transfer reactions with the unpaired electrons of transition metals and organic radicals, resulting in the sequential formation of superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and finally water.

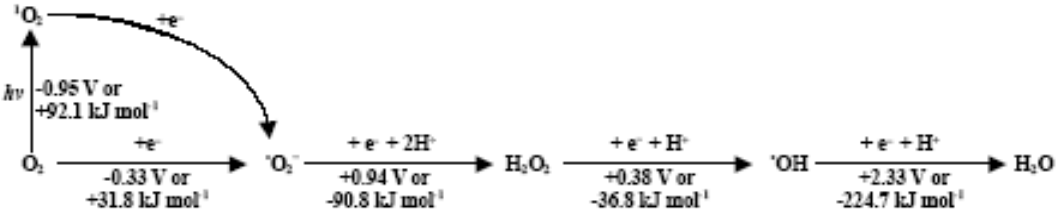


Fig.4: Pathways in the univalent reduction of oxygen to water leading to the formation of various intermediate reactive oxygen species (ROS). Numbers give approximate redox potentials (in volts) or the standard free energy of the reaction (in kJ mol^{-1}).

The first reduction step is free energy dependent (endergonic) and requires an electron donation, but the following one-electron reduction steps are exergonic and can occur spontaneously, using transition metal ions (Fe^{2+}, Cu^{+}) and semiquinones as electron donors.

The superoxide ($\cdot O_2^-$) produced during the first reaction is a short-lived ROS (approximately 2–4 μs) and not readily diffusible **(Smirnoff, 1993)**. In the cellular environment, $\cdot O_2-$ may cause lipid peroxidation, thus weakening cell membranes. The second reduction is an exergonic reaction that generates hydrogen peroxide (H_2O_2), a relatively long-lived (1 μs) and stable form of ROS. It can diffuse through membranes and therefore, reach cellular components distant from its site of synthesis **(Willekens *et al.*, 1997)**. The last ROS generated by this series of reductions is also exergonic and produces the highly reactive hydroxyl radical ($\cdot OH$), which is the most harmful form of ROS in plant tissues. It has a half-life of less than 1 ms and it has a very high affinity for biological molecules **(Dat *et al.*, 2000)**. Hydroxyl radical is generated from reaction between $\cdot O_2^-$ and H_2O_2 either spontaneously, by Haber-Weiss reaction (1), or in the presence of reduced transition metals, by Fenton reaction (2).

Under normal cellular conditions Haber-Weiss reaction (1) proceeds very slowly, and very low amounts of $\cdot OH$ are formed:

$$H_2O_2 + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}OH + OH^- + O_2 \quad (1)$$

In the Fenton reaction (2), the hydroxyl radical is also formed in very low amount. This reaction is common in biological systems, being Fe^{2+} and Cu^{+} in a chelated form, its transition metals.

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (2)$$

The availability of Fe^{2+} limits the rate of reaction, but Fe^{3+} can be efficiently reduced by superoxide, thus maintaining the Fenton reaction ongoing and leading to the generation of $^{\bullet}OH$ as shown in the two half reactions (3) and (4):

$$H_2O_2 + Fe^{2+} (Cu^{+}) \rightarrow Fe^{3+} (Cu^{2+}) + {}^{\bullet}OH + OH^- \quad (3)$$

$$^{\bullet}O_2^- + Fe^{3+} (Cu2+) \rightarrow Fe^{2+} (Cu^{+}) + O_2 \quad (4)$$

Therefore, the prevention of the Haber-Weiss (1) and Fenton (2) reactions can be reached when H_2O_2 and $^{\bullet}O_2^-$ are eliminated prior to these molecules get into contact with each other.

2.3.2 Sources of ROS

They are produced in response to both biotic and abiotic stresses such as drought, exposure to UV light, ozone, extremes in temperatures, elevated light intensity and pathogen challenge **(Bartosz, 1997**). The main sources of ROS under physiological conditions are respiration, photosynthesis and N_2 fixation (Fig. 5). Under most conditions, the Mehler reaction and photorespiration have the highest capacity to produce ROS (in the Mehler reaction, PSI reduces O_2 to $\cdot O_2^-$, superoxide dismutase (SOD) then converts it to H_2O_2 (**Foyer and Noctor, 2000).** In plants, ROS are produced in chloroplast, mitochondria, peroxisome, cytosol, plasma membrane and the apoplastic space. Cell wall bound peroxidases generate ROS in the apoplastic space. In the plasma membrane, the ROS-producing system is an NADPH oxidase **(Bolwell, 1999). Suzuki and Mittler, 2006** have also reported that ROS, such as superoxide ($\cdot O_2^-$), are

produced by NADPH oxidases during abiotic stress to activate stress-response pathways and induce defense mechanisms.

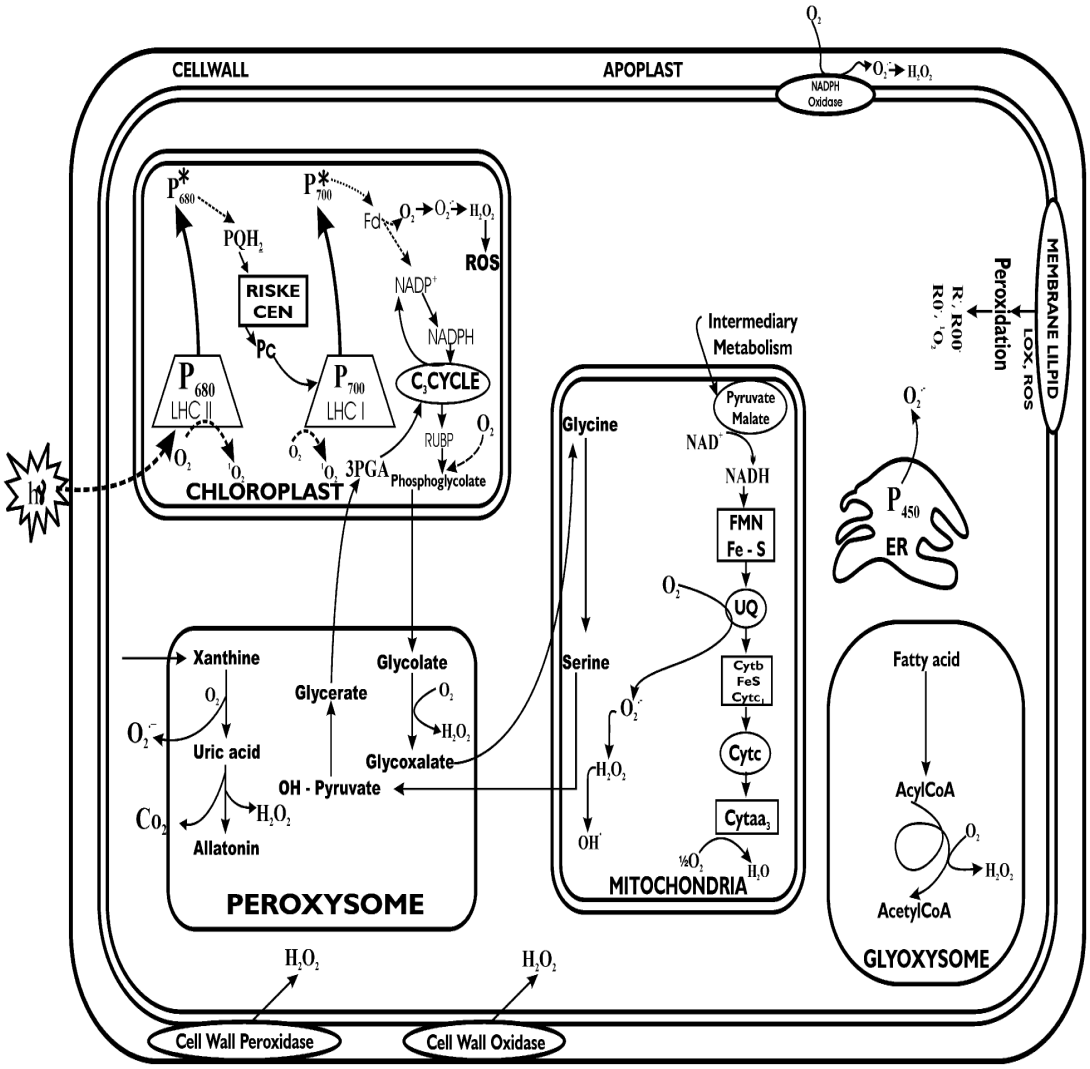


Fig. 5: Sources of ROS inside the cell

2.3.3 Hydrogen peroxide (H_2O_2)

H_2O_2 is the most stable ROS capable of behaving as an oxidant and as a reductant **(Salin, 1991).** ROS like H_2O_2 have multifunctional interactive roles in early stages of plant stress responses. H_2O_2 being a strong oxidant can initiate localized oxidative damage leading to disruption of metabolic function. H_2O_2 can also diffuse. Physiological concentrations (~10 μM) of H_2O_2 can oxidize thiol groups in RUBISCO,

glycerladehyde-3-phosphate dehydrogenase and fructose-bis-phosphotase, all three of which are chloroplastic enzymes involved in photosynthesis. As a result, the photosynthetic activity could be reduced by almost 50% **(Kaiser, 1979).** $\cdot O_2^-$ and H_2O_2 by themselves are relatively unreactive, but they can form species which are potentially damaging to essential cellular components. In the presence of metal ions (such as Fe^{2+}, Fe^{3+}), $\cdot O_2^-$ and H_2O_2 can react in a Haber Weiss reaction to form $OH^\bullet$ **(Hernandez *et al.*, 1993; Alscher *et al.*, 1997)**:

$$H_2O_2 + \cdot O_2^- \xrightarrow{Fe^{++},\ Fe^{+++}} OH^\bullet + O_2 + OH^-$$

$OH^\bullet$ radicals are among the most reactive species known in chemistry. $OH^\bullet$ can modify proteins so as to make them more susceptible to proteolytic attack **(Alscher *et al.*, 1997)**. They can also cause damage to all classes of biologically important macromolecules. The $\cdot OH$ can oxidize protein, mutagenize DNA and initiate lipid peroxide chain reactions leading to membrane disruption. These are perhaps the reasons why plants have developed an extensive array of antioxidants to remove ROS.

On the other hand, H_2O_2 was shown to play a major role in cellular processes as a signal, a mediator, and an effector molecule **(Levine *et al.*, 1994).** H_2O_2 is used by apoplastic peroxidases to reinforce cell wall and hinder pathogen penetration by catalyzing the cross-linking between cell wall extension and polysaccharides **(Sato *et al.*, 1993).** A role for H_2O_2 in the loosening of the cell wall has also been proposed by its ability to form $\cdot OH$ that is responsible for cell wall polysaccharide scission **(Schopfer, 2001).**

Thus, H_2O_2 increases mechanical strength and lowers the extensibility of plant cell walls. It is involved in the oxidative polymerization of cinnamyl alcohol to lignin (**Ros Barcelo, 1997).**

H_2O_2 is implicated to be one of the factors necessary for C3-CAM transition in *M. crystallinum* plants **(Slesak *et al.*, 2003).** H_2O_2 has been suggested to function in the openings of ion channels in guard cells in both ABA-dependent **(Pei *et al.*, 2000)** and ABA-independent pathways **(Kohler *et al.*, 2003)**. H_2O_2 can function as a secondary messenger in intracellular signaling pathways **(Finkel, 1998).** It induces expression of genes that are involved in pathogen response and chilling acclimation (**Chen *et al.*, 1993; Prasad *et al.*, 1994)**. It is one of the earliest factors involved in the transcriptional activation of defense related genes in Birch **(Pellinen *et al.*, 2002).** An internal signal that is positively regulated by H_2O_2 induces systemic oxidative bursts, which are in turn involved in rapid lesion formation leading to the development of hypersensitive response (HR). H_2O_2 is a systemic signal for APX2 induction in Arabidopsis **(Karpinski *et al.*, 1999).** In callus cultures of rice embryos, it transiently induces cytosolic APX **(Morita *et al.*, 1999).** Accumulation of H_2O_2 leads to a significant increase in APX and CAT activities within a few hours in root tips of Scots pine seedlings **(Schutzendubel *et al.*, 2001).** H_2O_2 induces expression of genes encoding GST and PAL **(Desikan *et al.*, 1998).** H_2O_2 can also induce expression of a DNA damage repair-protein (DRT112), a putative transcription factor (APK2b) and a LEA gene SAG21 in *Arabidopsis* cell suspension cultures **(Desikan *et al.*, 1999)**.

H_2O_2 may be involved in the induction of systemic acquired acclimation (SAA), *i.e.* exposure of one part of the plant to high light renders the unexposed parts resistant to high light **(Karpinski *et al.*, 1999)**. Rapid and often transient increase of H_2O_2 as a result of stress is thought to constitute a general alarm signal for subsequent triggering of durable defenses that are instrumental in mediating protection against these stresses **(Mittler and Berkowitz, 2001)**.

When the ROS production far exceeds the endogenous ROS-scavenging capacity, the regulated balance between the generation system and the scavenging system of ROS may be upset, leading to the inactivation of defence enzymes. Thus, $\cdot O_2^-$ and H_2O_2 must be completely scavenged to preserve photosynthetic activity. Although H_2O_2 is toxic to cells, it also plays important role in plants. Therefore, it is not entirely beneficial for plants to remove H_2O_2 as soon as it is produced, but rather to regulate its amount within the cell. APX is one such enzyme that may help regulate the amount of H_2O_2 in plant cells.

2.4 Antioxidative system and Plant defense

Plants have developed an elaborate defense system that includes antioxidant compounds and antioxidant enzymes to control and regulate the amounts of ROS in the cells. The term antioxidant is used to describe any compound that is capable of quenching ROS without itself undergoing conversion to a destructive radical. Ascorbic acid, α-tocopherol, flavonoids, glutathione and carotenoids are the non-enzymatic antioxidants that detoxify singlet oxygen and the hydroxyl radical.

Antioxidant enzymes catalyze reactions that remove ROS. The protective system of enzymes such as SOD, APX and CAT is a major ROS-scavenging mechanism of plants **(Asada and Takahashi, 1987; Bowler *et al.*, 1992; Willekens *et al.*, 1997)**. The activity and expression levels of the genes encoding detoxifying enzymes are probably enhanced by ROS under abiotic stresses. Transgenic plants over-expressing ROS scavenging enzymes, such as superoxide dismutase **(Alscher *et al.*, 2002)**, ascorbate peroxidase **(Wang *et al.*, 1999)**, and glutathione S-transferase/glutathione peroxidase **(Roxas *et al.*, 2000)** showed increased tolerance to osmotic, temperature, and oxidative stresses. These enzymes are capable of removing,

neutralizing, or scavenging free radicals and ROS. The pathways of ROS-scavenging in plants including SOD are found in almost all cellular compartments, the water-water cycle in chloroplast, the ascorbate-glutathione cycle in chloroplast, cytosol, mitochondria, apoplast and peroxisome, glutathione peroxidase, and CAT in peroxisomes. Fig. 6 shows the antioxidative network operated in different cell organelles.

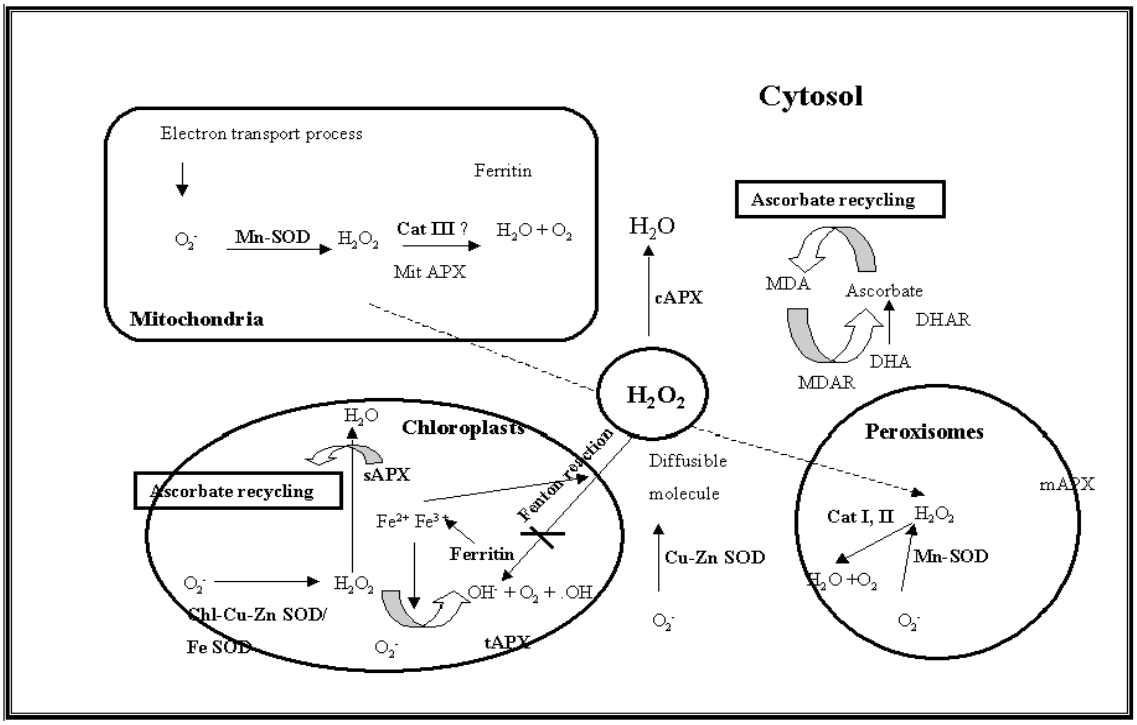


Fig.6 Antioxidant network in different organelles of the plant cell

2.5 Ascorbate-glutathione cycle

Ascorbate-glutathione cycle plays an important role in the detoxification of ROS in plant cell. In the first place superoxide dismutase converts $\cdot O_2^-$ to H_2O_2 and then ascorbate peroxidase uses ascorbate as its specific electron donor to reduce H_2O_2 to water with concomitant generation of monodehydroascorbate (MDHA), a univalent oxidant of ascorbate. Two moles of monodehydroascorbate

spontaneously disproportionate into one mole of dehydroascorbate (DHA) and one mole of ascorbate. Monodehydroascorbate can also be directly reduced to ascorbate by the action of NAD(P)H dependent monodehydroascorbate reductase (MDHAR). Dehydroascorbate reductase (DHAR) uses reduced glutathione (GSH) to reduce Dehydroascorbate and thereby regenerates ascorbate (Fig.7). The oxidized glutathione (GSSG) is then regenerated by glutathione reductase (GR) with the help of NAD(P)H, as reductant. AsA and GSH are not consumed in this pathways but participate in a cyclic transfer of reducing equivalents, involving four enzymes, which permit the reduction of H_2O_2 to H_2O using electrons derived from NAD(P)H. **(Noctor and Foyer, 1998)**.

This pathway is central to the oxidative defense strategy of the cell and is christened as **Ascorbate-Glutathione** pathway and many times is also referred to as **Halliwell-Asada** pathway.

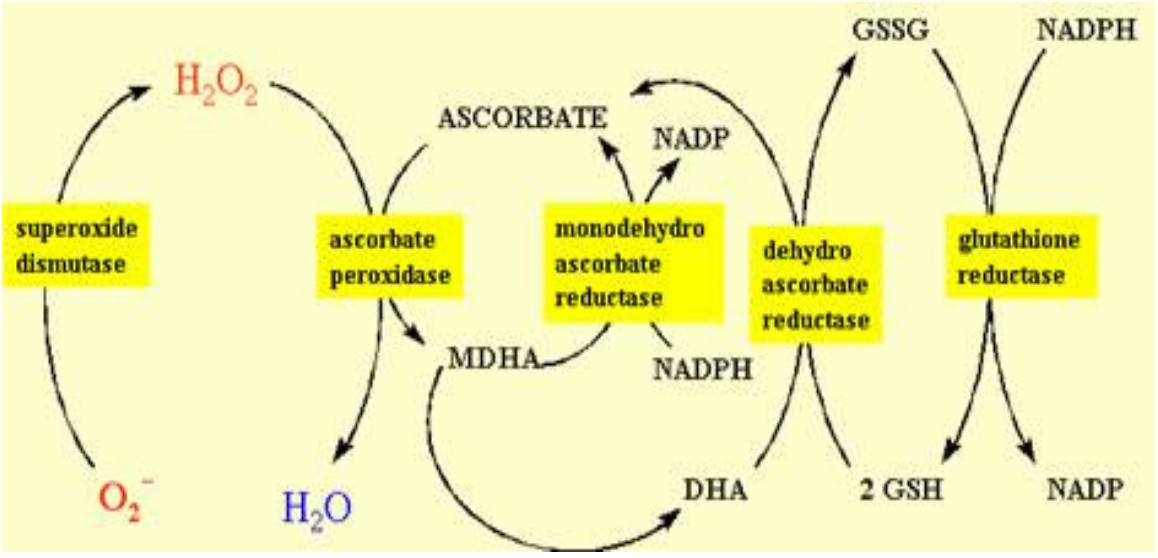


Fig.7: The Halliwell-Asada pathway

2.5.1 Ascorbate peroxidase (EC 1.11.1.11)

APX is a class I hemeperoxidase and was given the EC number1.11.1.11 based on the enzymatic properties of the Euglena

APX enzyme **(Shigeoka *et al.*, 1980)**. APX is found in Chlamydomonas **(Miyake *et al.*, 1991)**, Euglena **(Shigeoka *et al.*, 1980)**, Cyanobacteria **(Tel-Or *et al.*, 1986)** and in a red alga Galdieria partita **(Kitajima *et al.*, 2002)**. APX has also been found in the protozoan Trypanasoma cruzi **(Boveris *et al.*, 1980)** and insects **(Mathews *et al.*, 1997)**. It has never been reported from animals, except for one case from bovine eye **(Wada *et al.*, 1998)**. APX is more similar to yeast and bacterial peroxidase than plant peroxidases.

APX is the most efficient system or enzyme for scavenging smaller concentrations of H_2O_2 whereas at higher concentrations catalase is more effective [Km for catalase is 0.047 x 10^3 to 1.1 x 10^3 mM **(Halliwell, 1974)**, whereas Km for APX is 3 x 10^{-2}mM **(Nakano and Asada, 1987)**]. APX is more sensitive than SOD to H_2O_2 levels **(Drazkiewicz *et al.*, 2003)**. Nitric oxide (NO) can bind to the heme group of APX, thereby inhibiting its activity **(Clark *et al.*, 2000)**.

Crystal structure of recombinant pea APX has been determined at 2.2 A° resolution **(Dalton, 1996)**. **Wada *et al.* (2003)** have recently crystallized a cytosolic APX from tobacco and a 3-D structure model for this tobacco APX was proposed (Fig. 8).

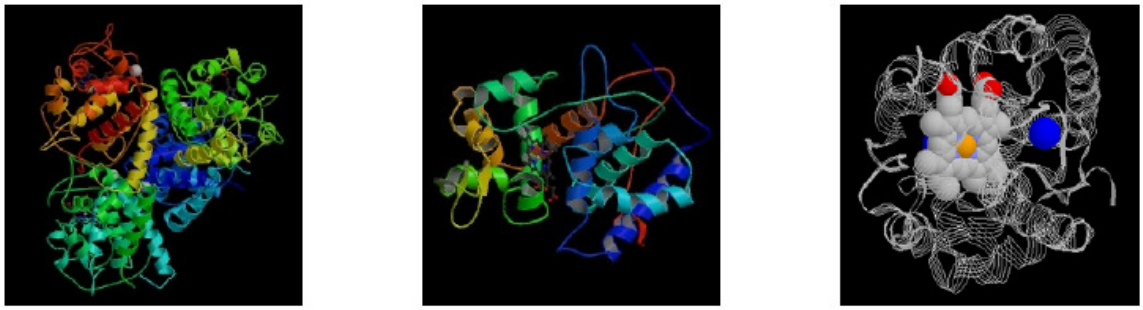


Fig.8: Predicted 3D structure of APX viewed from three different angles.

APX is distinct from guaiacol peroxidase (GPX), e.g. Horseraddish peroxidase (EC 1.11.1.7), in the following aspects **(Kubo *et al.*, 1992)**:

1. APX shows higher specificity for ascorbate
2. APX is not a glycoprotein
3. APX is inactivated under electron-donor depleted conditions
4. APX has a non heme iron in addition to protoheme
5. APX is inhibited by thiol reagents
6. Antibody raised against APX does not cross react with GPX and vice-versa
7. Neither nucleic acid sequence nor N-terminal amino acid sequence of APX resembles that of GPX
8. Four pairs of cystiene residues that form disulphide bonds in GPX are not present in APX

Enzyme characteristics such as substrate specificity, pH optimum lability in the absence of ascorbate, molecular mass, amino acid sequences and more importantly, subcellular localization differentiates plant APXs into five isoforms:

- cytosolic (molecualr mass 28 kDa)
- chloroplastic stromal (34 kDa)
- chloroplastic thylakoidal (38 kDa)
- glyoxysomal/peroxisomal (31 kDa) and
- mitochondrial.

The presence of APX in the apoplast has also been reported **(Castillo and Greppin, 1988)**. The cytosol and mitochondria of nodules have APX activity **(Dalton *et al.*, 1993; Iturbe-Ormaetxe *et al.*, 2001)** and it may count for up to 1% of the total soluble proteins in soybean nodules **(Dalton *et al.*, 1998)**. APX is localized to the infection region and the inner parenchyma cortex of bean and pea nodules **(Matamoros *et al.*, 1999)**. APX has very high substrate specificity for ascorbate (Km 300 µM for ascorbate and 20 µM for H_2O_2) and is the primary H_2O_2-scavenging enzyme in the chloroplast

and cytosol. Cytosolic APX has a reduced specificity for ascorbate compared to the chloroplastic form and a broader pH optimum **(Chen and Asada, 1989; Yoshimura *et al.*, 1998)**.

All peroxidases share similar catalytic cycle that has three irreversible steps and is often referred to as the 'peroxidase ping-pong' cycle **(Dunford and Stillman, 1976).** In the ping-pong reaction mechanism (below), the ferric enzyme APX is oxidized by H_2O_2, producing a 2-electron oxidized intermediate called compound I. Compound I oxidizes AA via two successive one-electron reactions, producing two molecules of MDA and is reduced to the resting ferric state.

$$APX(Fe^{3+})R + H_2O_2 \longrightarrow APX(Fe^{4+}=O)R^{\cdot} + H_2O \text{ (Compound I)}$$

$$APX(Fe^{4+}=O)R^{\cdot} + AA \longrightarrow APX(Fe^{4+}=O)R + MDA \quad \text{(Compound II)}$$

$$APX(Fe^{4+}=O)R + AA \longrightarrow APX(Fe^{3+})R + MDA + H_2O \text{ (Compound III)}$$

2.5.2 The gene encoding APX isoenzymes in higher plants

The cAPX genes from pea, *Arabidopsis*, tomato, and strawberry have been isolated and characterized **(Mittler and Zilinskas, 1992; Kubo *et al.*, 1993; Santos *et al.*, 1996; Kim and Chung, 1998; Gadea *et al.*, 1999)**. The pea cAPX gene (*APX1*) contains nine introns, the first of which is located within the 5'-untranslated region. Similar observations have also been made in those of other plant species, except for the *Arabidopsis APX1b* gene encoding a second family of cAPX. The expression of cAPX is regulated in response to several oxidative stress factors, indicating the presence of some functional *cis*-regulatory elements in the promoter. A putative heat-shock *cis*-element is present in the cAPX genes from all plant species and is associated with the *in vivo* induction of the gene (*APX1*) from *Arabidopsis* **(Storozhenko *et al.*, 1998)**. The expression of the

cAPX gene in rice is mediated by high temperature; furthermore, rice seedlings previously subjected to high temperature show increased tolerance to chilling stress **(Sato *et al.*, 2001)**. A functional G/C-rich element, which is essential for ethylene induction, is found in the APX1 promoter **(Storozhenko *et al.*, 1998)**. Furthermore, the leader intron in the 5'-untranslated region of the tomato cAPX gene is required to confer the constitutive gene expression **(Gadea *et al.*, 1999).**

The *APXII* gene, which encodes spinach chlAPX isoenzymes and is present in only one copy, consists of 13 exons split by 12 introns. The intron–exon splice junctions conform to the consensus sequences GT at the donor site and AG at the acceptor site **(Brown, 1986)**. Exons 1–11 encode the common amino acid sequence for sAPX and tAPX isoenzymes. The important point to note regards the two 3'-terminal exons. Two splice acceptor sites occur in exons 12 (penultimate) and 13 (final) separated by 14 bp nucleotides. The penultimate exon 12 consists of one codon for Asp-365 before the TAA termination codon and the entire 3'-untranslated region including a potential polyadenylation signal (AATAAA) of the sAPX mRNA. The final exon 13 contains the corresponding coding sequence of the hydrophobic C-terminal region, the TGA termination codon, and the entire 3'-untranslated region, including a potential polyadenylation signal (AATATA) of the tAPX mRNA. These facts show that the mRNAs for the chlAPX isoenzymes arise from only one gene by alternative use of the two final exons. The same mechanism has also been observed in the cases of pumpkin, *M. crystallinum*, and tobacco chlAPX isoenzymes, indicating that the alternative splicing of chlAPX isoenzymes is a common regulation mechanism in higher plants **(Mano *et al.*, 1997).**

2.5.3 Generation of chloroplastic APX isoenzymes by alternative mRNA splicing

By alternative processing of the 3'-terminal region as a result of alternative polyadenylation and splicing, four types of mature mRNA variants, one form encoding tAPX (tAPX-I) and three forms (sAPX-I, sAPX-II, sAPX-III) encoding sAPX, are produced as mature and functional forms, transported from the nucleus to the cytoplasm, and then incorporated into polysomes **(Yoshimura *et al.*, 1999)**. When the AAUAAA signal in exon 12 is selected, the only resulting product after the splicing of intron 11 should be sAPX-I mRNA. In contrast, the selection of the AAUAUA signal in exon 13 causes more complexity and, as a result, three mRNA variants (sAPX-II, -III, and tAPX-I) are produced by the alternative excision of intron 11 or intron 12 (Fig. 9).Alternative splicing is a common mechanism of gene regulation at the post-transcriptional stage in eukaryotic organisms **(McKeown, 1992; Simpson and Filipowicz, 1996).**

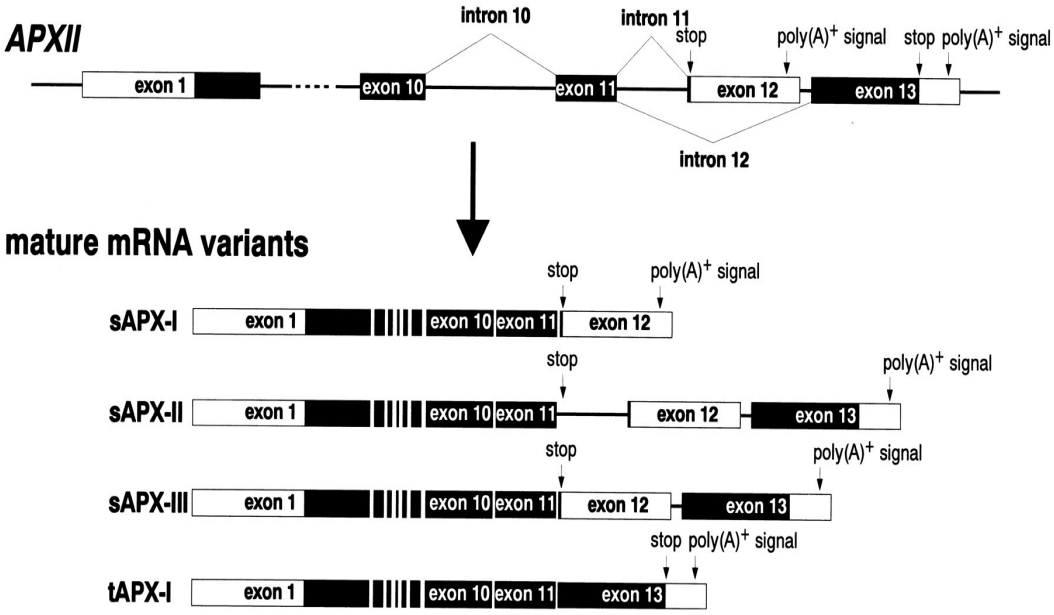


Fig.9: Alternative splicing patterns producing spinach chlAPX mRNA variants. Exon regions are shown as boxes and introns as lines. The open reading frame and untranslational regions are indicated by black and white boxes, respectively. Functional stop codons and polyadenylation signals are indicated.

2.5.4 Cytosolic APX

Cytosolic APXs (cAPXs) are encoded by a multigene family in Arabidopsis **(Santos *et al.*, 1996**). It is relatively easier to isolate the cytosolic isoforms from plant cells and that is perhaps why it has been reported from a number of plant species.

In Arabidopsis *APX1* and *APX2* encode the cytosolic isoform. *APX1* has 10 exons and nine introns. The first intron lies within the 5'-UTR, which may regulate the expression of *APX1*. The promoter region of *APX1* has ABA response elememt (ARE), Xenobiotic response element (XRE) and GPEI enhancer **(Mittler and Zilinskas, 1992)**. There is also a functional heat-shock element (HSE) in the promoter of *APX1* **(Storozhenko *et al.*, 1998)** and two HSEs in the promoter of *APX2* **(Santos *et al.*, 1996)**. The 5'-UTR of cAPX is required for constitutive expression in tomato (for leaves) and Arabidopsis (for roots) **(Gadea *et al.*, 1999; Fourcroy *et al.*, 2004).**

It has a very high preference for ascorbate and is specifically inhibited by hydroxyurea, p-chloromercurisulfonic acid, cyanide and azide **(Mittler and Zilinskas, 1991)**. In Maize, there is a greater concentration of cAPX in the mesophyll cells compared to the bundle sheath cells **(Pastori *et al.*, 2000)**. Cytosolic APX1 provides cross-compartment protection to chloroplastic APXs during light stress **(Davletova *et al.*, 2005)**.

In a study it was found that the lack of cytosolic APX1 resulted in the oxidation of chloroplastic proteins, suggesting that APX1 activity might be important for chloroplast protection (**Davletova *et al.*, 2005**). Previous studies have shown that chloroplasts are extremely sensitive to external application of H_2O_2 **(Asada, 2000)** and that chloroplastic APXs are inactivated by H_2O_2 **(Mano *et al.*, 2001)**. The lack of Cytosolic APX1 may therefore affect H_2O_2 scavenging systems in the chloroplast.

2.6 APX regulation during various abiotic stresses

All members of the APX gene family, irrespective of the isoforms they encode are ultimately associated with the functioning, stress responses, signaling processes and the development of chloroplast **(Mullineaux *et al.*, 2000)**. APX activity has been shown to increase in response to a number of stress conditions, such as drought **(Smirnoff and Colombe´, 1988; Tanaka *et al.*, 1990; Mittler and Zilinskas, 1994)**, air pollution **(Tanaka *et al.*,1985; Mehlhorn *et al.*, 1987; Conklin and Last, 1995; Kubo *et al.*, 1995; Rao *et al.*, 1996)**, high light intensity combined with chilling **(Schoner and Krause, 1990)** or deficiency in microelements **(Cakmak and Marschner, 1992)**, iron stress **(Vansuyt, *et al.*, 1997)**, excessive light (Karpinski et al.,1997), UV-B light **(Rao *et al.*, 1996)**, and salt stress **(Lopez *et al.*, 1996, Desingh and Kanagaraj, 2007)**. APX is inhibited in corn leaves under prolonged flooding but is activated under short term treatment **(Yan *et al.*, 1996)**.

Barley is relatively tolerant to salt stress, a higher APX activity is found in Barley under salt stress (**Khosravinejad *et al.*, 2008**). APX seems to be the key enzyme in determining salt tolerance in citrus; however the increase in APX is spatially different; high in the leaves and negligible in the roots **(Gueta-Dahan *et al.*, 1997)**. Under salt stress there is a significant difference between the activities of sAPX and tAPX in Pea. At 70-90 mM NaCl, the sAPX activity increased whereas the tAPX activity decreased **(Gomez *et al.*, 2004)**.

The isoforms that are induced seem to depend on plant species or varieties, physiological conditions of the plants and the type and extent of the oxidative stress given. There is a great degree of variance in literature with regard to the effect of altered antioxidant capacity

and stress resistance in plants. Both examples of increase in antioxidants correlated to resistance **(Alscher *et al.*, 1987)** and examples of increase in antioxidation causing an onset of visible injury **(Van Camp *et al.*, 1994)** are reported.

An increase in *APX1* and *APX2* expression is seen in Arabidopsis exposed to elevated light intensity (EL) (1000 μmol m^{-2} s^{-1}) **(Roseel *et al.*, 2002)**, although their patterns of expression differ. There is an 18-fold increase in the mRNA levels of *APX1* during EL and the levels are significantly higher during post stress. *APX2* transcript abundance increases within 7 minutes upon exposure to EL but is completely lost within 2 hours of post-stress **(Mullineaux *et al.*, 2000)**.

The expression of cAPX differs in different plant species subjected to different stresses. The mRNA levels of cAPX genes in spinach are regulated by oxidative stress **(Yoshimura *et al.*, 2000)** but not in wheat **(Danna *et al.*, 2003)**. On O_3 and SO_2 exposure, cAPX increases in *Arabidopsis* but not in tobacco **(Kubo *et al.*, 1995)**. **Orvar and Ellis (1997)** contradict the conclusion that tobacco cAPX may not play an important role in O_3 stress. In their study, antisense cAPX tobacco plants showed an increased susceptibility to ozone. cAPX increases in spinach in response to high light and methyl viologen (MV) treatment but the levels of sAPX, tAPX and pAPX do not change under these same stress conditions **(Yoshimura *et al.*, 2000)**.

It has been observed that cytosolic APX transcripts as well as cytosolic APX activities are increased after salt stress treatment in *Mesembryanthemum crystallinum*. The induction of APX clearly shows that this enzyme plays a critical role in controlling increase of H_2O_2 concentration in plant cells during the initial salt-induced oxidative stress **(Slesak *et al.*, 2002)**.

2.7 *Brassica*: A model system for Tissue culture and Genetic Engineering

Amongst all major food crops, members of the Genus *Brassica* have been very amenable to genetic manipulation for development of transgenic lines **(James, 1999).** *Brassica* species are particularly receptive to gene transformation techniques with other advantages as a small stature, short life cycle, self pollination and a small genome. *Brassica* species are closely related to *Arabidopsis* and represent ideal candidate for model-to-crop approaches as they include important crop plants.

Brassica juncea has several advantages compared to other *Brassicas* for

- improving salt tolerance like
- Better adapted to dry land conditions
- More resistant to diseases
- Due to its shorter growth period compared with *Brassica napus* and *Brassica carinata* , has proved more promising in semi arid and arid climates with short winters.

2.8 Relevance of transgenic approach to Abiotic stress tolerance

Recently, manipulation of the expression of enzymes, involved in scavenging of reactive oxygen species, by gene transfer technology has provided new insights into the role of these enzymes in abiotic stress tolerance. Recent attempts to modify and understand oxidative stress tolerance in plants, have involved the manipulation of enzymes associated with Halliwell-Asada pathway, including superoxide dismutase, ascorbate peroxidase and glutathione reductase. Over-expressing the key regulatory enzymes of the plant antioxidant

defense pathway will not only help to strengthen the antioxidative defense of the affected plant but also help us to understand the regulatory mechanisms controlling the activities of the antioxidant enzymes under stress.

Salinity is a multifactorial problem and the use or the breeding of salt-resistant crop varieties will require a clear understanding of the complex mechanisms of salt-stress resistance which is still lacking (**Sosa *et al.*, 2005**). Traditional breeding efforts made to introgression such complex traits from related salt tolerant germplasm have met with limited success. In the last five decades practical progress in breeding for salt tolerance has not been significant, salt tolerance is one of the least understood genetic traits and is considered intractable.

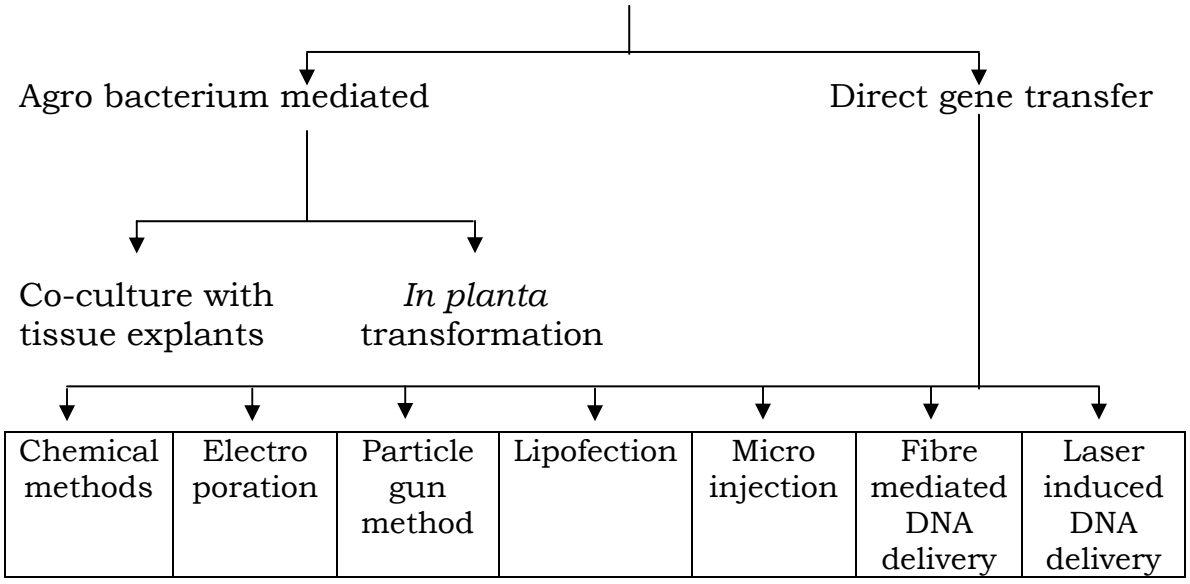
Improving salinity and drought tolerance of crop plants by genetic means has been an important but largely unfulfilled aim of modern agricultural development. However, rapid progress in understanding biochemical mechanisms that may participate in plant stress responses and salt tolerance as well as the molecular cloning of genes involved in the various metabolic pathways that respond to salt stress, offer new approaches to solve this persistent problem **(Bohnert and Jensen, 1996; Winicov and Bastola, 1997).**

The economic evidence available to date does not support the widely held perception that transgenic crops benefit only large farms; on the contrary, the technology may be pro-poor (**Ruttan, 2004**). Nor does the available evidence support the fear that multinational biotechnology firms are capturing all of the economic values created by transgenic crops. On the contrary, the benefits are shared by consumers, technology suppliers and adopting farmers.

2.9 Transformation techniques

There are many approaches devised for gene transfer.

These can be grouped into following two broad categories.



Only a few have been successful in a wide range of plant species. *Agrobacterium* mediated gene transfer (co-culture with tissue explants) exploits the natural gene transfer capacity of the bacterium, is the main method used for transformation of *Brassica juncea*.

2.10 *Agrobacterium*: a biological tool for transformation

Agrobacterium tumefaciens is well known for its ability to mediate transfer of DNA and proteins into the nuclei of plant cells, providing one of the best-studied examples of horizontal DNA transfer and the only known natural instance of interkingdom DNA transfer. In nature, *A. tumefaciens* detects a variety of chemical signal molecules released from plant wound sites and responds by expressing a battery of dedicated virulence (Vir) proteins, which are encoded by genes residing on the Ti plasmid. These proteins play various roles in the processing and transfer of a portion of the Ti plasmid, called the T-

DNA, into the plant cell. *A. tumefaciens* is a Gram-negative, non-sporing, motile, rod-shaped bacterium, closely related to *Rhizobium* which forms nitrogen-fixing nodules on clover and other leguminous plants. *Agrobacterium* genus has 4 species and belongs to Rhizobiaceae family (Fig. 10) along with the *Rhizobium* genus of nitrogen fixers**.**

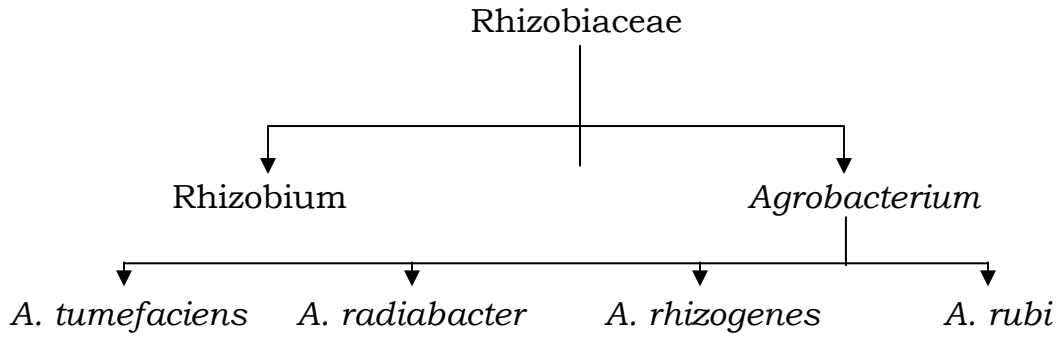


Fig.10. Phylogeny of *Agrobacterium*

The transformation of plant requires integration of transgenes. *Agrobacterium* are Ti plasmid-based vectors continue to offer the best system for plant transformation. An *Agrobacterium* cell acts the vehicle for a transforming plasmid. It contains a Ti plasmid with functional *Vir* genes for recognising plant signals and for excising the T-DNA to provide the T-DNA with appropriate deletions and gene inserts to the plant system.

Ti plasmid

The Ti plasmids are classified into different types based on the type of opine produced by their genes. The opines specified by pTi are octopine, nopaline, succinamopine and leucinopine. The different Ti plasmids can be grouped into two general categories: Octopine type and Nopaline type; .they differ mainly in the organisation of their T-DNAs The succinamopine and leucinopine specifying pTi are

similar to the nopaline type pTi, while octopine type of pTi forms a separate group. **Radchuk *et al.* (2000)** developed the procedure for genetic transformation of two spring and one winter rapeseed cultivars and found that Nopaline strains of Agrobacterium tumefaciens GV3101 and EHA105 were shown to be preferable for gene transfer, as compared to the octopine strain GV2260. The *A. tumefaciens* nopaline type strain GV3101 **(Zambryski *et al.*, 1983)** was found to give higher transformation frequencies in *B. juncea* than strain PGV 2260, LBA 4404 and A281 **(Jagannath *et al.*, 2003).**

2.10.1 *Agrobacterium* - mediated gene transfer

The current status of our understanding of *Agrobacterium* TDNA transfer process has been reviewed by **(Zupan and Gelvin, 2000).** The mechanisms governing the transfer of 'T-complex' via the conjugation channel and the roles of plant and Agrobacterium proteins in T-DNA integration are being intensely studied (Fig.11). *Agrobacterium*-based DNA transfer system offers many unique advantages in plant transformation:

1. The simplicity of *Agrobacterium* gene transfer makes it a 'poor man's vector.
2. A precise transfer and integration of DNA sequences with defined ends.
3. A linked transfer of genes of interest along with the transformation marker.
4. The higher frequency of stable transformation with many single copy insertions.
5. Reasonably low incidence of transgene silencing.
6. The ability to transfer long stretches of T-DNA (upto 150 kb).

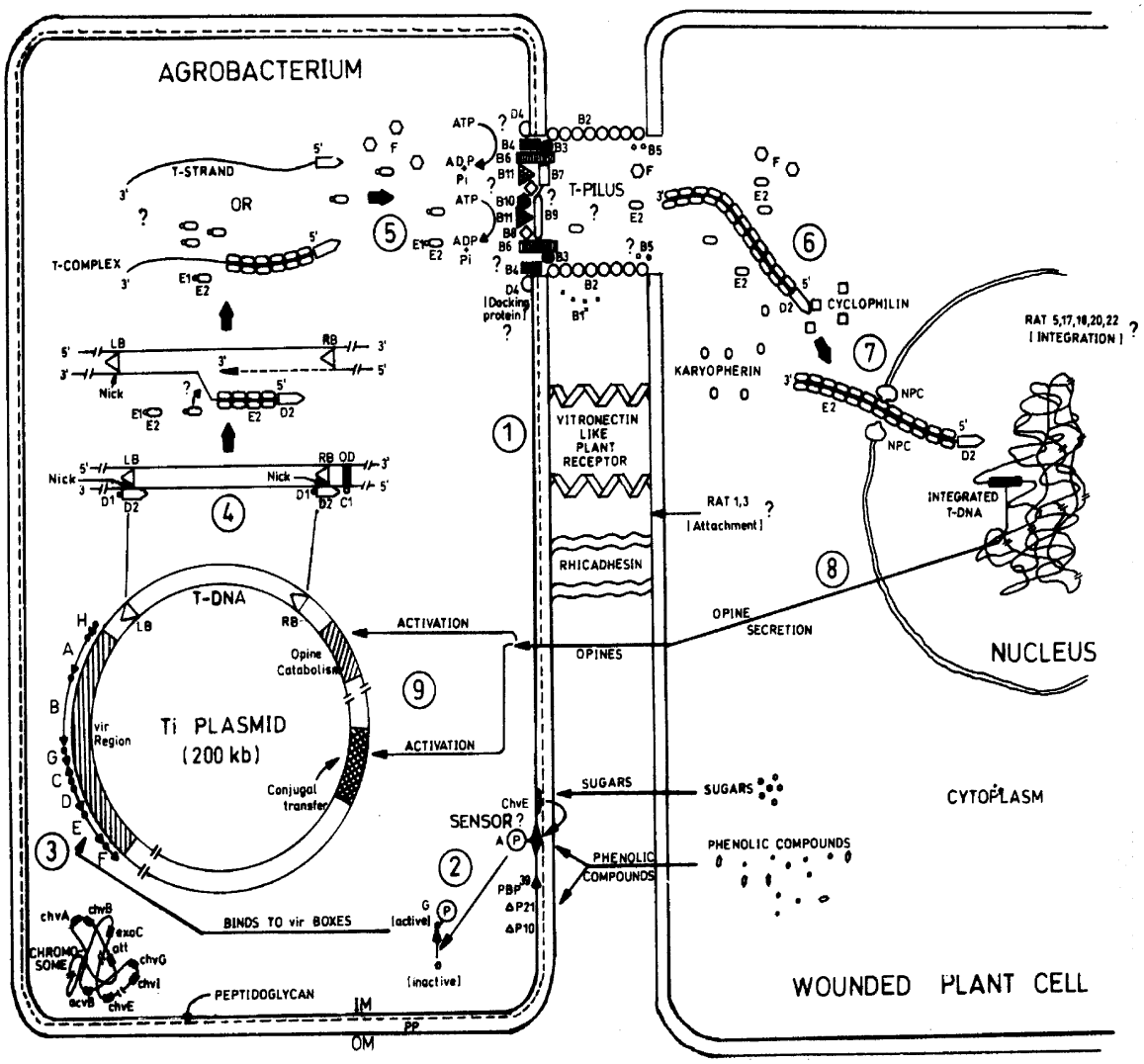


Fig. 11: A schematic diagram depicting the cascade of events leading to T-DNA transfer from *Agrobacterium* to the plant genome.

Transformation has been reported for all the main *Brassica* species, *B. oleracea* **(De Block *et al.*, 1989)**, *B. nigra* **(Gupta *et al.*, 1993),** *B. juncea* **(Barfield *et al.*, 1991),** *B. carinata* **(Narasimhulu *et al.*, 1992**) and *B. napus* **(Moloney *et al.*, 1989).** Protocols vary not only between the *Brassica* species but also within the species, with some genotypes being particularly recalcitrant to transformation. Transformation is therefore, highly genotype dependent. Factors that potentially make a genotype amenable to transformation are:

- **Susceptibility to *Agrobacterium***: Plant genotypes respond in a diverse way depending on the type and strain of *Agrobacterium* used **(Ramsay and Kumar 1990, Lindsey and Gallois, 1990)**. One reason for this is that some genotypes elicit a hypersensitive response in the presence of *Agrobacteria*. In extreme cases this can result in necrosis of the tissue at the infection site, hindering transformation events

- **Background antibiotic resistance**. The level of antibiotic required for selection of transformed cells varies between species. It is thought that genotypes differ in their natural background resistance to these antibiotics, and hence different levels of selection will be required for different genotypes.

- **Regeneration ability**. Again the ability to regenerate whole plant *in vitro* is highly genotype dependent, and is therefore likely to be under genetic control. **Murata and Orton, 1987** noted variation in regeneration ability between the Brassica species, with *B. oleracea* (CC) being the most responsive diploid followed by *B. nigra* (BB); whilst *B. rapa* (AA) was fairly recalcitrant to *in vitro* regeneration.

It is likely to be a combination of these factors that determines the transformability of individual genotype.

2.10.2 Vectors for use with *Agrobacterium*

Basically two types of vector systems have been developed based on the knowledge underlying the T-DNA transfer. Beside border all vectors should include a selectable marker for identification of transformed cell and bacterial selectable marker is also essential for introduction of the vector in *Agrobacterium* and subsequent selection **(Peralta *et al.*, 1986).**

Initially, co-integrative vectors, which offer plasmid stability in *Agrobacteirum* were extensively used; however, binary vectors have increasingly been adopted, primarily because of their ease of manipulation.

2.10.2.1 Co-integrative vectors

Co-integrative transformation vectors must include a region of homology between vector plasmid and the disarmed Ti plasmid. These are detection derivatives of the Ti plasmid that contain sequences, which allow the insertion of foreign DNA. This foreign DNA is cloned into an intermediate vector and that is used to transform the co-integrative vector by recombination within *Agrobacterium*.

2.10.2.2 Binary vectors

These are stable in *E. coli* and *Agrobacterium* and contain cloning site and marker gene flanked by the border sequences. Binary vectors allow manipulation to be made in *E. coli,* followed by transfer to *Agrobacterium* containing an active virulence (*vir*) region in helper plasmid, and used for plant transformation.

Binary transformation vectors contain origin of replication from a broad host range plasmid. A major advantage of binary vector is their lack of dependence on a specific Ti plasmid. Binary vectors also called 'trans' Ti gene vectors have been described by (**Bevan, 1984; Simones *et al.*, 1986).**

2.10.2.3 Selectable markers and reporter genes for plant transformation

In the recent years, several markers have been developed, in order to identify and select transformed plant cells, which have acquired foreign genes. These markers offer transformed plant cell a selective growth advantage over non-transformants, so that putative

transformants grow under a selection pressure (selectable markers), while normal non-transformed cells do not. The second types of markers make use of unique assayable enzymatic activities. These markers also provide unique nucleotide sequences that can be detected by Southern hybridization in putative transgenic tissues and normally confer antibiotic resistance.

A widely used selectable marker has the neomycin phosphotransferase type II (*npt II*) enzyme gene which was originally isolated from prokaryotic transposon Tn5. It detoxifies aminoglycoside compounds such as kanamycin by phosphorylation. Hygromycin phosphotransferase (*hpt*) gene is another aminoglycoside resistance gene, which is used as a selectable marker. This marker gene was originally isolated from *E. coli* which confers antibiotic hygromycin B resistance by inactivating the antibiotics, where *hpt* was used as a marker gene. A number of other antibiotics or herbicides are widely used as selectable markers in plant transformation (Table 2).

Table 2: Selectable marker genes for plant transformation

Marker gene	Enzyme encoded	Selective agent	Reference
Antibiotics			
nptII	Neomycin phosphotransferase II	Kanamycin Neomycin Geneticin (G418)	Bevan *et al.,* 1983 Fraley *et al.,* 1983 Herrera-Estrella *et al.,* 1983
hph, hpt	Hygromycin Phosphotransferase	Hygromycin B	Waldron *et al.,* 1985 Van den Elzen *et al.,* 1985
aacC3, aacC4	Gentamycin-3-Nacetyltransferase	Gentamycin	Hayford *et al.,* 1988
ble	not known	Bleomycin Phleomycin	Hille *et al.,* 1986

dhfr	Dihydrofolate reductase	Methotrexate	Herrera-Estrella *et al.,* 1983
SPT	Streptomycin Phosphotransferase	Streptomycin	Jones *et al.,* 1987
aadA	16S rRNA Aminoglycoside-3.-adenyltransferase	Spectinomycin Streptomycin	Svab *et al.,* 1990a Svab *et al.,* 1990b
Herbicides			
bar	Phosphinothricin Acetytransferase	Phosphinothricin	De Block *et al.,* 1987
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones	Haughn *et al.,* 1988
aroA	5-nolpyruvylshikimate -3- phosphate synthase	Glyphosate (Roundup)	Comai *et al.,* 1985 Shah *et al.,* 1986

2.11 Regeneration studies in *Brassica juncea*

The technique of *in vitro* cultivation of plant cells or organs, primarily denotes to ensure the described development in the cells and organs by providing suitable nutrient media and other environmental conditions which relies mainly on the manipulations of culture medium, especially growth regulators and to a much lesser extent on other factors including environmental conditions. In *Brassica juncea*, although cultured explants are recalcitrant to regeneration **(Fazekas *et al.*, 1986; Jain *et al.*, 1988; Narasimhulu *et al.*, 1988)**. Plants of *B. juncea* have been regenerated via *in vitro* organogenesis from protoplasts, **(Chatterjee *et al.*, 1985; Kao and sequin-Swartz, 1987; Bonfils *et al.*, 1992)** hypocotyls and cotyledon explants **(Hui and Zee, 1978; George and Rao, 1980; Sharma *et al.*, 1990).** It has been reported that shoot regenerability of *B. juncea* can be affected by the geographical origin, where the brown seeded Indian lines are generally more regenerative than the yellow seeded

Chinese/European lines **(Fazekas *et al.*, 1986).** In addition, the shoot regenerability of different *B. juncea* explants e.g. hypocotyls and cotyledons are greatly affected by the gelling agent of the medium, although plant regeneration from protoplasts originating from hypocotyls **(Kirti and Chopra, 1990)** and leaves **(Chatterjee *et al.*, 1985)** have been reported. Cotyledon and leaf explants usually regenerates well on medium solidified with 0.8 per cent Difco-Bacto agar, but the medium was inhibitory to shoot regeneration of hypocotyls explants **(Pua, 1993; Pua and Chi, 1993)**. The regenerability of hypocotyls could be markedly enhanced by replacing the agar with 0.4 per cent agarose **(Barfield and Pua, 1991).** Regeneration frequency increased when 0.7 per cent agarose was used as the gelling agent instead of agar agar **(Paul and Sikdar, 1999).**

Efficient production of multiple shoots often requires successful application of tissue culture techniques. An increased concentration of cytokinin and low auxin favours shoot bud induction **(Tisserat, 1985)**. Cytokinins are derivatives of adenine, which are responsible for its promotive growth or cytokinesis. Generally a medium containing zero or low auxin concentration is suitable for organogenesis. Cytokinins namely BAP, kinetin, zeatin and thidiazuron are required by plant cell with zero or low auxin concentration to induce adventitious shoot formation**.** The commonly used cytokinin in *Brassica juncea* tissue culture is BAP **(Mehra *et al.*, 2000; Bisht *et al.*, 2004)** and kinetin along with BAP **(Dhawan *et. al.*, 2004).**

2.12 Development of transgenic lines of *Brassica juncea*

Prasad *et al.* (2000) transformed *Brassica juncea* cv. Pusa Jai Kisan with *cod A* gene for choline oxidase from *Arthrobacter globiformis* with aim to introduce glycine betaine biosynthetic pathway for salinity stress tolerance.

Mehra *et al.* (2000) developed transgenic lines resistant to herbicide phosphinothricin (PPT) were developed in mustard (*Brassica juncea*). Seedling-derived hypocotyl explants were transformed with a disarmed *Agrobacterium tumefaciens* strain GV3101.

Jagannath *et al.* (2001) has developed male sterile lines (barnase/barstan) and storer lines for heterosis breeding. They have also developed cre/lox system for marker removal.

Huysen *et al.* (2003) overexpressed Cystathionine- c-synthase from *Arabidopsis thaliana* (L.) Heynh. in Indian mustard [*Brassica juncea* (L.) Czern & Coss], and five transgenic CGS lines with up to 10-fold enhanced CGS levels were compared with wild-type Indian mustard with respect to Se volatilization, tolerance and accumulation. The CGS transgenics showed 2- to 3-fold higher Se volatilization rates than wild-type plants when supplied with selenate or selenite.

Bisht *et al.* (2004) genetically transformed the hypocotyl explants derived from *Brassica juncea* cv. Varuna with *tfd A* gene. In transformation experiments, explants were infected with disarmed Agrobacterium strain GV 3101 containing the binary plasmid with *tfd A* gene. Shoots regenerated from the hypocotyls explants at an overall transformation frequency of 8.5 and 11.2 per cent in two different expriments. Transgenics were rooted in Kanamycin containing medium and maintained *in vitro* as nodal cultures.

Kanrar *et al.* (2006) cloned the FAE1 Fatty Acid Elongation1 (FAE1) homologue from *Brassica juncea* cv. Pusa Bold in a binary vector both in sense and antisense orientations under the control of the CaMV35S promoter. The recombinant binary vectors were used to transform *B. juncea* cv. RLM 198 via *Agrobacterium tumefaciens*.

Mondal *et al.* (2007) reported the expression of a class I basic glucanase gene, under the control of CaMV 35S promoter, in Indian mustard and its genetic resistance against alternaria leaf spot. In an

in vitro antifungal assay, transgenics arrested hyphal growth of *Alternaria brassicae* by 15–54%.

Xu *et al.* (2008) transformed the *Brassica juncea* with dehydrin genes; *BjDHN2* and *BjDHN3* and transgenic lines were found tolerant to salinity and freezing stress.

In the view of the above review, it can be inferred that genetic improvement of *Brassica juncea* with Cytosolic Ascorbate peroxidase, for strengthening the antioxidative defense mechanism, against salinity stress, could be of strategic importance in the varietal development of *Brassica juncea*. Such an attempt would also be extremely useful in understanding the details of the regulatory mechanisms controlling the expression of antioxidative enzymes, under stress.

Materials and Methods

Chapter 3 MATERIALS AND METHODS

The present investigations on the over-expression of *apx1* gene in *Brassica juncea* (*var*. pusa jaikisan) were carried out in the Department of Biochemistry, College of Basic Sciences & Humanities, G. B. Pant University of Agriculture & Technology, Pantnagar.

3.1 Materials

3.1.1 Plant Material

Seeds of *Brassica juncea* variety pusa jaikisan were obtained from National Research Centre for Rapeseed and Mustard (NRCRM), Bharatpur (Rajasthan) India.

3.1.1.1 Description of experimental material

3.1.1.1.1 Growth Habit

Brassica juncea (Indian mustard) is a cool season plant grown as an annual herb with rapidly emerging seedlings. The plant actually is a perennial herb, but is usually grown as an annual or biennial. It grows from 1 to 2 m in height and the plants cover the ground in 4 to 5 weeks under favorable moisture and temperature conditions. Plant height at maturity varies from 30 to 45 inches depending on type, variety and climatic conditions. Flower buds are visible about five weeks after emergence. Yellow flowers begin to appear 7 to 10 days later and continue blooming for a longer period with an adequate water supply. A longer flowering period increases the yield potential. Most of the flowers mature into approximately 2 inch long pods containing dark, reddish-brown seeds. (A detailed description of the plant is provided in the 'review of literature' part of this thesis).

3.1.1.1 Description of Variety pusa jaikisan

Parentage	:	Somaclone of Varuna
Year of release	:	1993
Developed by	:	National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi
Plant height	:	189 cm
Maturity	:	112-135 days
Oil content	:	40 %
Average reported yield	:	900 – 1150 kg/ha
Stress Tolerance	:	Not Specified

3.1.2 *Agrobacterium tumefaciens* Strain and Plasmid

The strain C58C1rifR pGV2260 **(Deblaere *et al.*, 1985)** containing ptpApx construct (Fig 3.1) was kindly provided by Dr. Dirk Inze, Department of Plant System Biology Unit, Ghent-Belgium.

The *Agrobacterium tumefaciens* strain C58C1rifR pGV2260 is an octopine type strain which comprises of an octopine type helper plasmid in a C58 bacterial background with a disarmed Ti plasmid. pGV2260 contains the intact vir region which encodes Ti plasmid functions necessary for T-DNA transfer and/or integration. These functions can act *in trans* to the T-DNA **(Hoekema, 1983)**.

The construct ptpApx was prepared in the background C58C1 (pGV2260). The plasmid size is 12.094 kb. Base plasmid of the construct is pBR and pVS1, Where CaMv35S promoter was fused with APX cDNA insert. The cDNA for Cytosolic APX (*apx1*) was isolated from *Arabidopsis thaliana*, which shares a homology of 70 per cent with *Brassica juncea* at the genetic level. The construct ptpApx carried

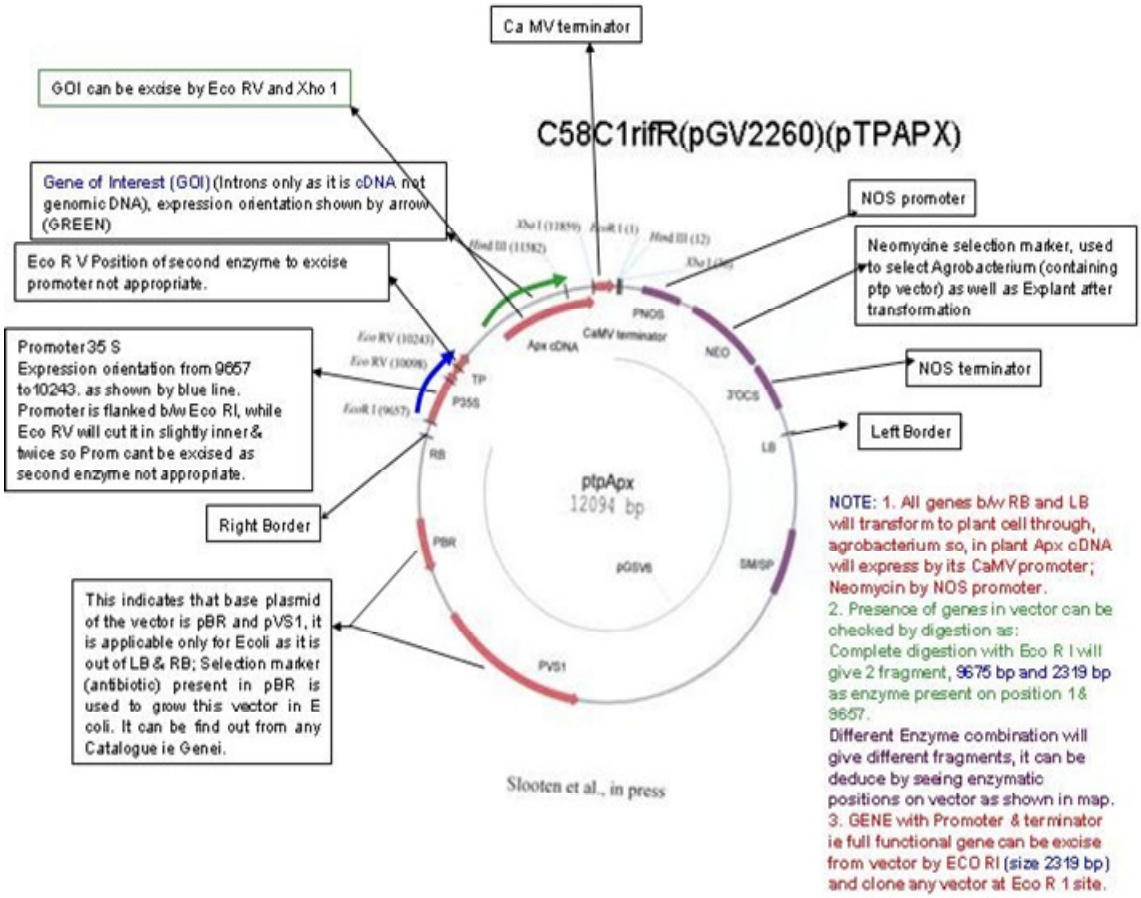


Figure 3.1: Plasmid map of ptpApx

plant selectable marker gene, the neomycin phosphotransferase-II (*nptll*) gene controlled by the nopaline synthase promoter and terminator sequences. C58C1 is rifampycin resistant, pGV2260 is resistant to carbenicillin and ptpApx is resistant to streptomycin and spectinomycin. Fig 3.2 show the details of *Apx1* construct used in experiment.

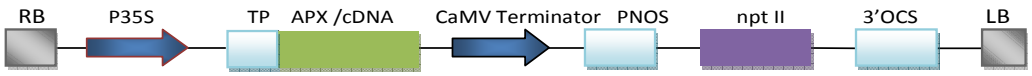


Fig3.2: Details of *apx1* gene construct used. Schematic representation of T-DNA regions of the binary vector that carried gene for Cytosolic ascorbate peroxidase. cassette is flanked at the 5' end by the 35S promoter (P35S) and at the 3' end by OCS terminator. 35S-P, 35S promoter of cauliflower mosaic virus; TP, transit peptide for cytosol; PNOS ,promoter of nopaline synthase; *nptII*, gene for neomycin phosphotransferase OCS; octopine synthase terminator; RB, right border; LB, Left border.

3.2 Plan of work

The complete course of investigation was divided into following sections:

1. Regeneration and transformation studies, using hypocotyl explants of *Brassica juncea var*. pusa jaikisan. *Brassica juncea* was transformed with cytosolic Ascorbate peroxidase (*apx1*) gene construct, through *Agrobacterium* mediated genetic transformation technique.
2. Physiological, biochemical and molecular analysis of putative *Brassica juncea* transgenic plants.
3. Evaluation of transgenic plants over-expressing cytosolic Ascorbate peroxidase under salinity induced oxidative stress.

3.3 Methodology

3.3.1 Plant Regeneration studies

3.3.1.1 Composition of Plant Tissue Culture Medium

Murashige and Skoog (MS) medium (1962) supplemented with graded combinations of plant growth regulators BAP and NAA was used for shoot regeneration and rooting of differentiated shoots. Composition of MS medium along with recipe for preparation of stock solutions and preparation of final working medium is given in table 3.1. Stock solutions of different plant growth regulators, used in the present study were prepared as described in table 3.2. All the stock solutions of MS medium were stored at 4^0C in the refrigerator and required quantities were pipetted out at the time of media preparation.

Table 3.1 Composition of Plant Tissue Culture (MS) Medium

Stock A (20X)

Constituents	Conc.(1x) (mg/l)	Stock Conc. (g/l)	g/500 ml	Amount used for 1L of 1X
KNO_3	1900	38	19	
NH_4NO_3	1650	33	16.5	
KH_2PO_4	170	3.4	1.7	50 ml
$MgSO_4.7H_2O$	370	7.4	3.7	
$CaCl_2.2H_2O$	440	8.8	4.4	

Stock B (200 X)

Constituents	Conc.(1x) (mg/l)	Stock Conc. (g/l)	g/100 ml	Amount used for 1L of 1X
$ZnSO_4$	8.6	1.72	0.172	
H_3BO_3	6.2	1.24	0.124	
KI	0.83	0.166	0.0166	
$MnSO_4$	22.3	4.46	0.446	5 ml
Na_2MoO_4	0.25	0.05	0.005	
$CuSO_4$	0.025	0.005	0.0005	
$CoCl_2$	0.025	0.005	0.0005	

Stock C (200X)

Constituents	Conc.(1x) (mg/l)	Stock Conc. (g/l)	g/100 ml	Amount used for 1L of 1X
Na_2 EDTA	37.3	7.46	0.746	
$FeSO_4.7H_2O$	27.8	5.56	0.556	5ml

Stock D (200X)

Constituents	Conc.(1x) (mg/l)	Stock Conc. (g/l)	g/100 ml	Amount used for 1L of 1X
Pyridoxine HCL	0.5	0.1	0.01	
Thiamine HCL	0.1	0.02	0.002	5 ml
Nicotinic Acid	0.5	0.1	0.01	
Glycine	2.0	0.4	0.04	
Myo-inositol	100	20.0	2.0	

Table 3.2 Preparation of Plant Growth Regulators

	Growth regulator	Stock (mg/ml)	Solvent	Storage temp.
(i)	***Auxins***			
	NAA(Naphthalene cetic acid)	1.0	0.1N NaOH*	4°C
	IBA (Indole butyric acid)	1.0	0.1N NaOH*	4°C
(ii)	***Cytokinins***			
	BAP (6-Benzyl amino purine)	1.0	0.1N NaOH*	4°C

* The plant growth regulators were dissolved in minimum volume of 0.1 N NaOH and the final volume was made up by adding deionized water

3.3.1.2 Preparation of culture media

Stock solutions were mixed sequentially in the required volume to get the working (1x) concentration of the MS constituents. Different

concentrations of plant growth regulators, BAP and NAA, in combination were added in the medium, in a 'checker board' fashion (Table 3.3), to screen for the combinations which could generate maximal a) shoot induction and b) callus induction response. The pH of the medium was adjusted to 5.8 (using 0.1 N NaOH), after the addition of sucrose (30 g/l). Double distilled water was used to make up the final volume; thereafter 0.7 % (w /v) agar powder was dissolved by boiling the solution.

Culture bottles were thoroughly cleaned with soap solution and rinsed with distilled water and dried in a hot air oven and then autoclaved. About 35-40 ml of medium was poured in each bottle, which was then autoclaved at 15 psi for 20 minutes at 121°C.

Table 3.3: Checker Board combination of different concentrations of BAP and NAA for multiple shoot induction and callus induction response.

BAP ▶ / NAA ▼	0.5 mg/l	1.0 mg/l	1.5 mg/l	2.0 mg/l	2.5 mg/l
0.5 mg/l	B-0.5 N-0.5	B-0.5 N-1.0	B-0.5 N-1.5	B-0.5 N-2.0	B-0.5 N-2.5
1.0 mg/l	B-1.0 N-0.5	B-1.0 N-1.0	B-1.0 N-1.5	B-1.0 N-2.0	B-1.0 N-2.5
1.5 mg/l	B-1.5 N-0.5	B-1.5 N-1.0	B-1.5 N-1.5	B-1.5 N-2.0	B-1.5 N-2.5
2.0 mg/l	B-2.0 N-0.5	B-2.0 N-1.0	B-2.0 N-1.5	B-2.0 N-2.0	B-2.0 N-2.5
2.5 mg/l	B-2.5 N-0.5	B-2.5 N-1.0	B-2.5 N-1.5	B-2.5 N-2.0	B-2.5 N-2.5

3.3.1.3 Sterilization

3.3.1.3.1 Sterilization of glassware and Accessory material

Glasswares were cleaned with liquid soap, washed under running tap water and finally rinsed with distilled water. These were sterilized in oven by incubating them for 2 hours at 120^0C. The Scalpel, forceps and glass petriplates and pipettes were cleaned with

absolute alcohol and wrapped in aluminum foil, which were sterilized by autoclaving them at 15 psi for 20 min at 121°C in a pressure cooker type autoclave.

3.3.1.3.2 Sterilization of Media

Conical flasks and culture tubes containing MS growth media were plugged with non-absorbent cotton and wrapped with paper. They were then autoclaved at 15 psi for 20 min, at 121˚C.

3.3.1.3.3 Sterilization of Laminar air flow chamber

The surface of laminar airflow chamber was cleaned with sprit. All the required materials (except the living plant tissues and microorganisms) were kept inside the laminar air flow chamber. Filtered air (@ 80 to 100 ft./minute) was allowed to flow for 5 minutes to ensure that particles do not settle in the working area and subsequently ultraviolet light (UV) was switched on for 30 min. After 30 min. of UV treatment, the tissue culture work involving transfer, cutting and inoculation of plant tissue etc. was done near the gas burner flame inside the chamber under fluorescent light with filtered air flowing all the time.

3.3.1.4 Seed sterilization and inoculation

For seed germination, the seeds of *Brassica* juncea (*var.* pusa jaikisan) were initially washed with a mild detergent for five minutes and then washed under running water to flush out soap and sticky dust. Surface sterilization of seeds was done with 0.1% mercuric chloride ($HgCl_2$) for approximately two minutes. The seeds were then thoroughly washed three times with autoclaved distilled water inside a laminar air flow. Washed seeds were soaked in autoclaved distilled water for six hours in dark. Twelve seeds per bottle were placed on jellified MS growth medium, with the help of sterilized forceps. All the

steps of inoculation were performed in the laminar air flow under aseptic conditions. After inoculation, the bottles were kept in the tissue culture incubation chamber at a temperature of 26±1 ^{0}C and a relative humidity of ~ 70%. Illumination was provided by four 40W fluorescent tubes having a photon flux density of approximately 21,500 lux with a 16h/8h: day/night cycle.

3.3.1.5 Callus Induction and shoot regeneration from hypocotyl explants

In the present experiments hypocotyls of *Brassica juncea* (*var.* pusa jaikisan) were used as explants for the regeneration studies. The hypocotyls from 6 days old seedlings of *Brassica* seedlings were aseptically cut into 0.8 to 1.0 cm long hypocotyl pieces. The explants were aseptically placed on MS basal media supplemented with different combinations of BAP and NAA. The hypocotyl pieces in petriplates were incubated under cool fluorescent light (21,500 lux) with 16 hours photoperiod. Observations for callus induction, callus growth, callus with shoots and direct multiple shooting from explants, were taken at regular intervals. Percent callus induction frequencies (% CIF) and Percent shoot induction frequencies (% SIF) were calculated as follows:

$$\%\,CIF = \frac{\text{Number of calli producing explants}}{\text{Total number of explants in the culture}} \times 100$$

$$\%\,SIF = \frac{\text{Number of shoots producing explants}}{\text{Total number of explants in the culture}} \times 100$$

3.3.1.6 Sub-culturing

After 3 weeks of culture, shoot primordia emerged from the cut ends of hypocotyls. The regenerated shoots were given sharp cut and

transferred to the multiple shoot induction medium containing 2.5mg/l BAP. The transferred shoots were allowed to grow in multiple shoot induction media for 6 weeks with regular sub-culturing after 21 days.

3.3.1.7 Rooting of differentiated shoots

The differentiated shoots about 3 cm in length were cut and carefully transferred into MS basal medium (MS Constituents + 1.8 % Sucrose + 0.8 % agar) supplemented with increasing concentrations (0.1, 0.5, 1.0 and 1.5 mg/l) of Indole butyric acid (IBA). Observations for per cent root induction frequency (% RIF) were taken after 2 weeks of transfer to different root induction media.

$$\%\, RIF = \frac{\text{Number of Shoots producing roots}}{\text{Total number of shoots inoculated in root induction media}} \times 100$$

3.3.1.8 Acclimatization of regenerated plantlets

The *in vitro* rooted plantlets were taken out from the culture vessels and the roots were carefully washed with running tap water so as to remove pieces of agar sticking to the plantlets. Next they were planted in disposable plastic cups containing coco-peat. They were immediately watered and kept covered with perforated poly bags in hardening chamber of transgenic glass house under controlled temperature (25±2°C) and high relative humidity (~70%). After two weeks the polybags were removed and the plantlets were grown for another 2 weeks under similar conditions. After that, plants were transferred to plantation pots containing *garden soil: sand: vermin-compost* in a 2:1:1 ratio and grown to maturity.

3.3.2 Transformation Studies

3.3.2.1 Preparation of antibiotic stocks

Six different antibiotics were used for selection during various stages of transformation studies. Stock solutions of Kanamycin acid

sulphate, Carbenicillin, Cefotaxim, Streptomycin and Spectinomycin were prepared by dissolving them directly in sterile distilled water (Table 3.4). Rifampicin was prepared by dissolving in minimal volume of 50% ethanol and the required volume was made up with sterile distilled water. All antibiotic stock solutions were filter sterilized by filtering them through sterile 0.22 µm Millipore filter and were stored in refrigerator at 4^0C for further use. Complete details of the antibiotics used during the transformation experiments are given in table 3.4.

Table 3.4 Antibiotics used during transformation studies

Antibiotics	Working Conc.	Stock Conc.	Role
Rifampicin	100 µg /ml	100 mg/ml	Chromosomal marker for *Agrobacterium* strain
Carbenicillin	100 µg /ml	100 mg/ml	Helper plasmid marker for *Agrobacterium* strain
Streptomycin	100 µg /ml	100 mg/ml	For selection of *Agrobacterium* strain harbouring ptpApx.
Spectinomycin	100 µg /ml	100 mg/ml	For selection of *Agrobacterium* strain harbouring ptpApx.
Kanamycin	10-30 µg /ml	100 mg/ml	For the selection of transformants.
Cefotaxime	300 mg/l	100 mg/ml	Bacteriostatic agent, inhibits the growth of *Agrobacterium.*

3.3.2.2 Preparation of Yeast Extract Broth (YEB) medium

YEB medium was used to culture Agrobacterium tumefaciens strain C58C1RIF^R pGV2260. It is considered a rich medium for supporting the growth of Agrobacterium. The composition of the medium is as given in table 3.5.

Table 3.5 Composition of YEB media

	Component	Quantity (g /L)
A	Yeast extract	1
	Beef extract	5
	Peptone	5
	Sucrose	5
	$MgSO_4.7H_2O$	0.5
B	YEB semi-solid	+ 1.5% (w/v) Agar
C	YEB Gel for stab culture	+1.2% (w/v) Agar

The pH of liquid medium was adjusted to 7.2 with 1 N NaOH and 20 ml of liquid YEB medium was dispensed in conical flasks/culture tubes of 100 ml capacity and then autoclaved. Semi-solid YEB agar plates were prepared by adding agar (1.5% w/v) to the required volume of liquid medium. After autoclaving the medium was allowed to cool to a temperature of about 50-55^{0}C and then requisite antibiotics viz. streptomycin, spectinomycin and rifampicin were added in desired quantities.

3.3.2.3 Culture and maintenance of *Agrobacterium tumefaciens*

Broth culture of Agrobacterium was prepared by inoculating a single colony in 20 ml YEB medium with appropriate concentration of antibiotics [streptomycin (100µg/ml), spectinomycin (100µg/ml) and rifampicin (100µg/ml)] and incubating the inoculants at 28^{0}C @ 110 rpm in an orbital shaker. The Agrobacterium culture was routinely maintained and revived at one month interval, on petriplates having gelified YEB medium, with antibiotics.

3.3.2.4 Preparation of glycerol stock

Equal volume of 40% glycerol (autoclaved) and overnight grown culture of *Agrobacterium tumefaciens* (log phase) in liquid YEB

medium containing appropriate concentration of antibiotics, was mixed in an eppendorf tube and vortexed properly to evenly disperse the glycerol. The tubes were sealed with parafilm, labeled and stored at –80°C.

For revival of the strain, glycerol stock was streaked on YEB plates, using a sterile inoculation needle. The plates were sealed with parafilm and incubated at 28°C for two days in dark in a BOD incubator.

3.3.2.5 Preparation of growth curve

A Single colony of *Agrobacterium tumefaciens* strain pGV2260 was inoculated in 20 ml liquid YEB medium containing Streptomycin (100µg/ml) and Spectinomycin (100µg/ml) antibiotics. The inoculated media was incubated at 28°C @ 110 rpm for 22-24 hours, for developing primary culture. 100 µl of this primary culture was inoculated in each of the 72 tubes containing 5 ml YEB broth. All tubes were incubated at 28°C @ 110 rpm and absorbance of bacterial culture was recorded at every 2 hours interval till 36 hours. Graph was plotted between the absorbance and time interval.

3.3.2.6 Plasmid DNA isolation from Agrobacterium:

3.3.2.6.1 Reagents required

1. **Antibiotic solutions (Table 3.4)**
2. **YEB medium**
3. **Solution I** (Autoclaved and stored at room temperature)

   [50 mM glucose; 25 mM Tris HCl (pH = 8.0); 10 mM EDTA (pH = 8.0)].
4. **Solution II (Freshly prepared)**

   [0.2N NaOH ; 1% SDS]

5. **Solution III (pH =5.2)**

   For preparation of 100 ml solution III, following were dissolved.

   5 M potassium acetate 60.0 ml

   Glacial acetic acid 11.5 ml

   H_2O 28.5 ml

   Autoclaved Double distilled water was used to make up the final volume to 100 ml.

6. **Lysozyme** (20 mg/ml in 10 mM Tris-HCl, pH 8.0)

7. **Tris saturated phenol (pH 8.0)**

8. **Phenol: Chloroform: isoamyl alcohol (25:24:1)**

9. **$T_{10}E_1$ (10 mM Tris and 1 mM EDTA, pH = 8.0)**

10. **TAE (50 X)**

    24.2 g of Tris base, 5.71 ml of glacial acetic acid and 10.0 ml of 0.5 M EDTA (pH = 8.0) were dissolved in 55 ml of distilled water. Volume was made up to 100 ml with distilled water. The solution was autoclaved and stored at room temperature. This stock solution was diluted 50 times to obtain 1X concentration prior to use for preparation and running of agarose gel for DNA electrophoresis.

11. **Agarose**

12. **Ethanol (95 %)**

13. **Isopropanol**

14. **RNase Solution**

    RNase A was dissolved at a concentration of 10 mg/ml in 0.01 M Tris-Cl (pH 8.0) and heated to 100^0C for 15 min and cooled slowly to room temperature. It was dispensed into aliquots and stored at –20 ^{0}C.

15. **Ethidium bromide [(10 mg/ml) stock solution]**

Ethidium bromide powder (10 mg) was dissolved in 1.0 ml sterile distilled water and stored at 4^0C in an eppendorf tube covered with Aluminum foil. This solution was diluted to have 0.5 µg/ml concentration for staining the agarose gel after run.

16. **Gel loading dye**

For preparation of 1 ml of gel loading dye, 0.0025 g bromophenol blue and 300 µl of glycerol and 100 µl of 0.1 M Tris.Cl (pH = 8.0) were dissolved in 500 µl of sterile distilled water. The volume was made up to 1ml with sterile distilled water and stored in eppendorf tube at 4^0C after proper mixing.

3.3.2.6.2 Protocol for Plasmid DNA Isolation

1. Single colony of Agrobacterium tumefaciens was inoculated in 50 ml of YEB media containing Streptomycin (100µg/ml) and Spectinomycin (100µg/ml) antibiotics. The culture was incubated at 28^0C @ 110 rpm in an orbital shaker for 14 hrs (log phase).
2. The culture was centrifuged at 10,000 rpm for 10 min.
3. Bacterial pellet was resuspended in 1 ml of solution I.
4. The lysozyme solution (200 µl) was added and mixed well, the mixture was incubated at 37^0C for 15 min.
5. To the above solution, 2 ml of cell lysis buffer (Solution II) was added and mixed completely by repeated gentle inversion of tube.
6. After gentle mixing, 500-µl Tris saturated phenol (pH = 8.0) was added and vortexed, to mix it completely.
7. 2 ml of neutralization solution (Solution III) was added and mixed with slight inversion of the tube. The mixture was centrifuged at 10,000 rpm for 10 min.
8. The upper aqueous phase was transferred to another tube and 0.7 volumes of isopropanol was added to it and kept at -20^0C for overnight precipitation.

9. The solution was centrifuged at 12,000 rpm for 5 min to spin down the DNA/RNA pellet.
10. Pellet was washed with 70 % ethanol and air dried.
11. Finally the pellet was resuspended in 100 µl TE buffer.
12. To the DNA dissolved in TE, 3 µl of RNase stock solution (10 mg/ml) was added and incubated at 37 ^{0}C for 60 minutes.
13. After incubation, equal volume of phenol: chloroform: Isoamyl alcohol (25: 24:1) was added and resultant solution was mixed by inversion and gentle mixing and then centrifuged at 10,000 rpm for 5 min.
14. The aqueous phase was collected in a fresh tube and 1/10 th volume of 5 M potassium acetate (pH 5.2) was added along with double volume of isopropanol.
15. The mixture was kept at – 20^{0}C for 2 hours.
16. The mixture was centrifuged at 12,000 rpm for 5 min to spin down the DNA pellet.
17. Pellet was washed with 70 % ethanol and air dried.
18. The air-dried pellet was dissolved in 50 µl TE buffer.
19. Agarose gel (0.8 % w/v) was prepared in 1X TAE buffer.
20. 10 µl of TE dissolved plasmid solution was mixed with 2 µl of gel loading dye and loaded on to the gel.
21. The gel was run at 50 volts for 2 hours and results checked in a gel documentation system.

3.3.2.7 Quantification of DNA

The DNA sample (10 µl) was added to 490µl TE, and mixed by inverting the tube. The absorbance was read in spectrophotometer by setting the blank against TE. The absorbance was taken at 260 nm and 280nm. The ratio of Abs_{260}nm/Abs_{280} nm provides an estimate of

purity of nucleic acid. Pure preparation of DNA has Abs_{260}/Abs_{280} ratio of 1.8 to 2.0.

DNA concentration was calculated by using the following formula:

$$DNA \text{ concentration } (\mu g/\mu l) = \frac{Abs_{260} \times \text{Dilution factor} \times 50}{1000}$$

Where,

$$\text{Dilution factor} = \frac{\text{Total volume of sample}}{\text{Volume of the DNA sample taken from stock}}$$

3.3.2.8 Confirmation of presence of apx1 gene insert in the vector through PCR

The presence of target apx1 cDNA insert in the vector was confirmed through PCR amplification of specific sequences. The sequence of forward and reverse apx1 and nptII primers used in the present investigations are as follows:

nptII Primers

Direction	**5'-Po**	**Tm**	**Primer Sequence (5'->3')**	**Product Length**
Forward	9	56	AGATGGATTGCACGCAGG	734
Reverse	742	56	AGCGGCGATACCGTAAAG	

apx1 Primers

Direction	**5'-Pos**	**Tm**	**Primer Sequence (5'->3')**	**Product Length**
Forward	79	56	GGCTGTTGAGAAGTGCAG	898
Reverse	976	54	CAGCAAAAGCGCAACGG	

The lyophilized primers were dissolved in autoclaved double distilled water and 100 µM stock of oligonucleotide was prepared (Dissolve lyophilized primers in 'x' volume of deionized water; where x = (number of nmol of primer X 10) µl). Then add 10 µl from 100 µM stock solution to 90 µl of autoclaved double distilled water to get final working concentration of 10 µM.

3.3.2.8.1 Colony PCR

The presence of *apx1* cDNA carrying plasmid was confirmed through colony PCR. Single colony of *Agrobacterium* was taken as template DNA in PCR reaction. The constituents of the polymerase chain reaction were as follows:

A. PCR constituents

Sl. No.	Components (Conc. Of Stock)	Volume for one reaction	Final Concentration
1.	Sterile double distilled water	18.0 µl	
2.	dNTPs mix (10 mM)	1.0 µl	2.5 mM
3.	Primers (Forward) (10 µM)	1.0 µl	2.5 µM
4.	Primers (Reverse) (10 µM)	1.0 µl	2.5 µM
5.	Assay buffer (10 x)	2.5 µl	1 x
6.	Taq DNA polymerase (3.0 U/µl)	0.5 µl	1.5 U
7.	Template DNA	1 Colony	
	V_f =	~ 25.0µl	

B. PCR conditions

Following reaction conditions were used for optimal amplification of apx1 and nptII gene inserts:

Steps	Temperature	Time
Initial denaturation	94^{0}C	5 min
Denaturation	94^{0}C	1 min
Annealing	54^{0}C	1 min
Extension	72^{0}C	2 min
Final extension	72^{0}C	7 min
Total No. of Cycles = 35		

3.3.2.9 Standardization of Transformation Protocol

The following parameters were studied in order to develop an efficient and reproducible transformation protocol for Brassica juncea var. pusa jaikisan.

3.3.2.9.1 Kanamycin sensitivity test of hypocotyl explants

In order to select appropriate concentration of kanamycin at which transformed hypocotyl explants or emerging shoots could be selected, kanamycin sensitivity test of explants was carried out. Hypocotyl explants, derived from 6-day-old *Brassica juncea* seedlings were placed on to basal MS medium with selected plant growth regulator concentration (2.5 mg/l BAP and 0.5 mg/l NAA) along with 3% sucrose and supplemented with different concentrations of kanamycin sulfate (0, 10, 20, 30, 40 and 50 mg/l). Data for growth of explants at different concentrations was recorded after 7, 14 and 21 days after inoculation.

3.3.2.9.2 Cefotaxime sensitivity of *Agrobacterium tumefaciens*

On the basis of previous studies from our laboratory, in the present investigation, 300 mg/l cefotaxime was used to arrest the growth and multiplication of *Agrobacterium* **(Singh *et al.*, 2006)**.

3.3.2.10 Transformation media

Different growth media used during transformation and plantlet regeneration studies are described in Table 3.6.

Table: 3.6 Different media used in transformation experiments

Media used for preculturing	
PCM (pre culture medium)	→ MS constituents + BAP (2.5 mg/l) + NAA (0.5 mg/l) + 3% (w/v) sucrose
Washing medium (WM)	→ Autoclaved double distilled water + 300 mg/l cefotaxime

Media used for regeneration and selection		
Shoot regeneration medium (*SR*)	→	MS constituents + 2.5 mg/l BAP + 0.5 mg/l NAA + 3% (w/v) sucrose + 20 µM $AgNO_3$ + 0.8% agar
Selection cycle		
SR_1	→	*SR* + 20 mg/l kanamycin + 300 mg/l cefotaxime + 20 µM $AgNO_3$
SR_2	→	*SR* + 20 mg/l kanamycin + 300 mg/l cefotaxime + 20 µM $AgNO_3$
SR_3	→	*SR* + 30 mg/l kanamycin + 300 mg/l cefotaxime + 20 µM $AgNO_3$
Root induction medium (RI_1)	→	MS constituents + 0.5 mg/l IBA + 20 mg/l kanamycin + 3% (w/v) sucrose + 0.8% agar

3.3.2.11 Steps for Agrobacterium mediated genetic transformation of *Brassica juncea* explants

a) Preparation of Agrobacterium culture

Primary culture : Primary culture of Agrobacterium was prepared by inoculating single colony of Agrobacterium in 20 ml YEB medium supplemented with streptomycin (100µg/ml), spectinomycin (100µg/ml) and rifampicin (100µg/ml) antibiotics and incubated at 28^0C @ 110 rpm for 48 hours in dark.

Secondary Culture : For secondary culture, 300 µl of primary culture was inoculated in 30 ml YEB without antibiotics and grown at 28^0C @ 110 rpm till $Abs._{600}$ reached 0.5 to 0.6.

The culture was taken in an oakridge tube and centrifuged at 6000 rpm for 15 min. Pellet was dissolved in the PCM medium so as to dilute it to an final $Abs._{600}$ = 0.2 - 0.3. This Agrobacterium suspension was used for infection and co-cultivation of hypocotyl explants.

b) Preparation of explants

Six days old Brassica seedlings were taken out in sterile petri dishes. They were aseptically cut to remove cotyledonary leaves and root portion. Hypocotyl's proximal to node were taken as experimental material. Hypocotyls were cut into 0.7 - 1cm long segments and were used as explants.

c) Pre-culture of explants

The explants were immediately transferred to PCM, to prevent drying. To avoid over-teasing of explants proper care was taken, not to injure the explants. For acclimatization, the explants were pre-cultured in liquid PCM medium for 48 hours in a rotary shaker at 28^0C @ 110 rpm in dark.

e) Infection

The hypocotyl explants were suspended in the Agrobacterium suspension (Abs. = 0.2-0.3) for 30 min under sterile conditions. After that, they were pipetted dry.

f) Co cultivation

After infection, the explants were co-cultivated for 24 hours at 28^0C @ 110 rpm by placing them in preculture medium (Table 3.6).

g) Washing of hypocotyl explants and inoculation on shoot induction media

After Co cultivation, the hypocotyl explants were washed thrice for 20 minutes each with WM (autoclaved double distilled water +

300 mg/l cefotaxime) with continuous shaking. Washed explants were blotted dry and placed in petri plates containing SR_1 medium. Plates were incubated under cool fluorescent light (21,500 lux), with a 16/8 hour light/dark photoperiod.

3.3.2.12 Selection and regeneration of transformed plants

The explants growing on SR_1 medium for 20 days were then transferred to SR_2 selection medium also containing 20 mg/l kanamycin. The surviving explants after 2 weeks were transferred to SR_3 selection medium containing 30 mg/l kanamycin.

3.3.2.13 Multiple shoot induction from putatively transformed shoots

For multiple shooting, the shoot apices were given sharp cuts and transferred to multiple shooting media containing MS constituents along with 2.5 mg/l BAP, 200 mg/l cefotaxime, 30 mg/l kanamycin, 3% sucrose and 0.8% agar. Care was taken to remove all the callus tissue from the base of the shoots, at the time of transferring to multiple shooting media.

3.3.2.14 Rooting of putatively transformed shoots

The well developed shoots, of approximately 3 cm length, were transferred to the root induction medium containing 15 mg/l kanamycin and christened as selected cycle 4 (SR_4 in Table 3.6). In the root induction media, BAP was not added and NAA was replaced by IBA, as it was found that IBA gives better root induction response than NAA.

3.3.2.15 Acclimatization of transgenic *Brassica juncea* plantlets

Hardening of rooted plantlets was done as described in section 3.3.1.8. The seeds from transgenic *Brassica juncea* plants (T_o) generation were collected and stored in a desiccator for further analysis.

3.3.3 Confirmation of Transformation Events

3.3.3.1 Leaf disc assay

Leaf disc assay was used as a diagnostic marker for nptII expression. For this leaf discs from green regenerated shoots of putative transformants were carefully removed. Selected leaf sections were placed onto the surface of MS basal medium containing 20 mg/l kanamycin. The culture plates were sealed and incubated under fluorescent light (21,500 lux) at 24-25^{o}C. Observations were made after one week of incubation.

3.3.3.2 DNA isolation from Brassica juncea plantlets

Total genomic DNA was isolated from young leaves of putatively transformed, kanamycin resistant plantlets as well as from the tissue culture grown wild type seedlings.

3.3.3.2.1 Solutions

1. **DNA extraction buffer (100 ml):**

Chemical / Stock	Final Concentration	Amount to be added for 100 ml
β-mercaptoethanol	0.2% (w / v)	1785 µl
CTAB	2.0 % (w /v)	2.0 g
Tris-Cl ,1.0 M, pH = 8.0	100 mM	10 ml
NaCl	1.4 M	8.12 g
EDTA (0.5 M)	20 mM	4 ml

2. **Chloroform: isoamylalcohol (24: 1)**
3. **5 M potassium acetate, pH 5.2**
4. **Isopropanol**
5. **70 % Ethanol**
6. **$T_{10}E_1$ buffer (pH 8.0)**

3.3.3.2.2 DNA extraction protocol

1. 1 g leaf tissue was rapidly frozen in liquid nitrogen and ground to fine powder in a pre-chilled sterile pestle and mortar.
2. The frozen powder was transferred to a 30 ml autoclaved oakridge tube.
3. 3 ml of CTAB extraction buffer was added to each tube. The tubes were inverted several times with gentle shaking and leaf powder was suspended in CTAB buffer.
4. The tubes were incubated at 65°C in a water bath for 60 min. Contents were mixed gently by inverting the tubes after every 10-15 minutes.
5. The tubes were cooled down to room temperature and 3 ml of chloroform: isoamyl alcohol in the ratio of 24: 1 was added to each tube. The contents were mixed gently by inverting the tubes several times.
6. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C.
7. The upper clear aqueous supernatant was removed and transferred into fresh tubes.
8. Steps 5-7 were repeated 2 times to ensure that there was no more protein contamination left.
9. 2 ml of prechilled isopropanol was added to each tube and the contents were mixed by inverting the tubes several times and the tubes were incubated overnight at -20°C. Although DNA precipitation began as soon as isopropanol was added, but it was kept overnight for complete precipitation, to maximize yield.
10. The samples were centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was poured out without disturbing the DNA pellet at the bottom of the tubes.
11. 1.5 ml of 70 per cent ethanol was added to wash the pellet and then centrifuged at 5,000 rpm for 5 min at 4°C.

12. Supernatant was decanted. Pellet was air dried for 15-20 min.
13. 200 µl of TE buffer was added to the pellet and the pellet was dissolved.

3.3.3.2.3 DNA Purification: RNase A Treatment

Procedure

1. 10 µl of RNase A (10 mg/ml) was added to the tube containing 200 µl of extracted DNA dissolved in TE buffer
2. The tubes were incubated for 30 min at 37°C in a water bath.
3. 40 µl of ice cold 3M sodium acetate was added to the DNA solution; followed by 250 µl of phenol: chloroform: isoamyl alcohol in the ratio of 25: 24: 1. The contents were mixed by inverting the tubes.
4. The samples were centrifuged at 6,000 rpm for 5 min at 4°C.
5. The upper aqueous phase was transferred to new tubes.
6. 250 µl of chloroform: isoamyl alcohol (24: 1) was added to the collected supernatant.
7. The suspension was again centrifuged at 6,000 rpm for 5 min at 4°C.
8. The upper aqueous phase was transferred to fresh tubes and 500 µl of prechilled ethanol was added to it. Genomic DNA precipitated immediately after inverting the tubes (for optimizing yield the solution should be incubated overnight at -20˚C).
9. The samples were centrifuged at 6,000 rpm for 5 min at 4°C. The supernatant was removed carefully and the pellet was air dried.
10. The DNA pellet was resuspended in 100 µl of TE buffer (pH 8.0) and the samples were stored at 4°C for further use.

3.3.3.2.4 DNA Quantification

The DNA samples were quantified as described earlier in section 3.3.2.8

3.3.3.3 PCR analysis of putative transformants

After selection of putative transformed shoots on media containing upto 30 mg/l kanamycin, the shoots were tested for stable integration of apx1 transgene through PCR using nptII and apx1 gene specific primers.

The sequence of forward and reverse npt II and apx1 primers used in the investigation are as listed in section 3.3.2.9

The genomic DNA was diluted to a final concentration of 50ng/µl.

PCR constituents

Sl. No.	Components (Conc. Of Stock)	Amount for one reaction mixture (µl)	Final Conc.
1.	Sterile water (55 M)	17.0 µl	-
2.	dNTPs mix (10 mM)	1.0 µl	2.5 mM
3.	Primers (F) (10 µM)	1.0 µl	2.5 µM
4.	Primers (R) (10 µM)	1.0 µl	2.5 µM
5.	10 x assay buffer	2.5 µl	1 x
6.	DNA polymerase with $Mgcl_2$ (3.0U/µl)	0.5 µl	1.5 U
7.	Template DNA (50 ng/µl)	2.0 µl	100 ng
	V_f	**25.0 µl**	

3.3.4 Physiological Studies

To compare the physiological status and growth of transgenic plants with that of wild type plants, following parameters were measured:

3.3.4.1 Photosynthetic rate and stomatal conductance

Rate of photosynthesis (μ mol CO_2 m^{-2} s^{-1}), was determined with the help of portable CO_2 gas analyzer. At the beginning of each measuring day, the infrared gas analyzer (IRGAs) was calibrated as per the manufacturer's instructions and the fluorometer signal offset was zeroed. The flow rate of air through the sample chamber was set at 0.4 LPM and leaf temperature was maintained at 27 ± 0.8°C by the chamber thermoelectric coolers. It was measured in an open system in which the instrument takes reference CO_2 from atmosphere. The response time and added interval time were, 15 sec and 20 sec, respectively. The area of window of leaf chamber (broad rectangular) was 11cm^2. The time of measurement was kept constant. Photosynthetic rate and stomatal conductance were measured on intact leaves. All readings were taken in full sun light and data was taken in triplicates for each treatment.

3.3.4.2 Chlorophyll fluorescence:

Chlorophyll 'a' fluorescence in green plants reflects the efficiency of photosynthetic PS-II system. A hand held plant efficiency analyzer (Handy PEA, Hansatech, UK) was used to evaluate the Fv/Fmax ratios in wild type and transgenic plants. Measurements were recorded in the forenoon hours (9-10 am) to avoid photo inhibition, due to excessive sunlight. The minimal fluorescence level (F_0) with PS II reaction centre was determined by measurements taken under modulated light, so as not to induce any significant variable fluorescence. The maximum fluorescence level (Fm) of closed PS-II centre was determined by providing 1.5 sec. saturating pulse at 300 μ mol m^{-2} s^{-1} on dark adopted leaves (20 minutes), by the following protocol:

1. Leaf clips were attached to mid portion of each leaf

2. Leaves were dark adapted for 20 minutes.
3. The fluorometer probe was connected to leaf clip holder.
4. Chlorophyll fluorescence measurement using red light (1.5 sec, 300 μ mol m^{-2} s^{-1})
5. Fv/Fmax ratios were recorded from the PEA meter directly.

3.3.4.3 Soil Plant Analysis Development (SPAD) Measurements:

The Chlorophyll meter, used for soil plant analysis development measurements, is a compact instrument used for measuring the chlorophyll content of plant leaves without damaging the plant. It also gives an indication of the nitrogen status of the plants. The measured chlorophyll content is an indication of the plant's condition and can be used to compare the growth and development status of plants growing under different treatments. The instrument measures transmission of red light at 650 nm, at which chlorophyll absorbs light and transmission of infrared light at 940 nm, at which no absorption occurs. On the basis of these two transmission values the instrument calculates a SPAD (Soil Plant Analysis Development) value that is quite well correlated with chlorophyll content. SPAD readings were recorded by a portable SPAD meter (Opti Science, CMM-200, USA) in sunlight.

3.3.5 Salinity Stress Tolerance Profiling of Transgenic *Brassica juncea*

3.3.5.1 Salinity treatments

Leaf discs of 1cm diagonal length were subject to salinity stress by floating them on half strength MS media supplemented without (0mM) and with 200 mM NaCl. Leaf discs from transformed and wild type Brassica juncea plantlets were placed in Petri dishes with covers, with each containing around 10 discs floated in 20 ml of control and 200 mM NaCl medium, and placed under continuous light at 26°C for 24 h.

These leaf discs were used as experimental material for assessing different standardized biochemical markers for salinity stress tolerance in transgenic as well as wild type *Brassica juncea* plantlets. All treatments were replicated three times.

3.3.5.1.1 Biochemical Parameters

3.3.5.1.1.1 Free Proline Content

Free proline was determined by the method of **Bates *et al.* (1973)**. One gram of leaf material was homogenized in 10ml of sulfosalicylic acid (3% w/v in distilled water) and the homogenate was centrifuged at 10000xg for 20 minutes at room temperature. 2ml of supernatant was mixed with 2ml of glacial acetic acid and 2ml of acid ninhydrin reagent. The reaction mixture was incubated for one hour at 100^0C in a water bath. After incubation the reaction was terminated in an ice bath. To each reaction mixture tube, 4ml of toluene was added and the chromophore was extracted by vigorous stirring on a vortex mixer. The absorbance of chromophore containing toluene layer was measured at 520nm. Concentration of proline in the sample was computed from a standard curve of L-proline. Results were expressed in µg of free proline gm^{-1} fresh weight.

3.3.5.1.1.2 Malondialdehyde content

The procedure of **Heath and Packer (1968)** was followed for measuring the MDA content. 0.1g of leaf material was homogenized in 1ml of 0.25% (w/v) 2-thiobarbituric acid (TBA) prepared in 10% (w/v) trichloroacetic acid (TCA).The homogenate was incubated at 95^0C for 30 minutes and was then centrifuged at 12000x*g* for 30 minutes, the supernatant was collected and allowed to cool at room temperature. Absorbance of the supernatant was measured at 532nm and 600nm. Absorbance at 600 nm is subtracted from the absorbance at 532nm

for non-specific absorbance. The concentration of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

3.3.5.1.1.3 Hydrogen peroxide content

Hydrogen peroxide was measured spectrophotometrically after reaction with potassium iodide (KI) **(Alexieva *et al.*, 2001)**. 0.2g of leaf was crushed in 1.0ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10000g for 30 minutes at 4^0C. The reaction mixture consisted of 0.5ml of supernatant, 0.5ml of 0.1M potassium phosphate buffer and 2ml of 1M KI reagent. The blank consisted of 0.1% TCA in place of leaf extract. The reaction was allowed to develop for 1 hour in dark and absorbance was measured at 390nm. The amount of H_2O_2 was calculated using standard curve prepared with different dilutions of a working standard of 100µM of H_2O_2.

3.3.5.1.1.4 Chlorophyll and Carotenoid content

Chlorophyll content was determined by the method of **Hiscox and Israelstam (1979)**. Fresh leaves (100 mg) were kept in the extraction reagent, dimethylsulphooxide (DMSO). The tubes were kept in the oven at 65°C for 40 min. Absorbance was determined photometrically at 470, 645, 663 nm using DMSO for a blank.

The *Chla*, *Chlb* and total chlorophyll (mg g^{-1} FW) concentrations in the leaf tissues were calculated according to the following equations:

$$\textbf{Chl a} = [(12.7 \times A_{663}) - (2.63 \times A_{645})] / (\text{wt. in gms} \times 1000)$$

$$\textbf{Chl b} = [(22.9 \times A_{645}) - (4.48 \times A_{663})] / (\text{wt. in gms} \times 1000)$$

$$\textbf{Total Chlorophyll} = [(20.2 \times A_{645}) + (8.02 \times A_{663})] / (\text{wt. in gms} \times 1000)$$

For carotenoid content (µg g^{-1} fr.wt.) following formulae **(Cha-um *et al.*, 2004)** was applied

$$[C_{X+C}] = [1000 \times A_{470} - 1.9\ (Chl_a) - (Chl_b)]/214$$

Chlorophyll stability index calculated by dividing chlorophyll content in leaf sample of stressed plants by chlorophyll content in leaf sample of control plants, it was expressed in percentage **(Deshmukh *et al.*, 1991).**

3.3.5.1.2 Anti-oxidative enzyme analysis

3.3.5.1.2.1 Preparation of extracts

For determination of specific antioxidant enzyme activities, 0.5g of leaves were homogenized in chilled 1.5ml of respective extraction buffer in a pre-chilled mortar and pestle using liquid nitrogen. The homogenate was centrifuged at 22,000×*g* for 20 min at 4˚C. The supernatant was re-centrifuged again at 22,000×*g* for 20 min at 4˚C for determination of antioxidant enzyme activities. Protein concentration of the enzyme extract was determined according to **Bradford (1976).**

3.3.5.1.2.1.1 Ascorbate peroxidase (APX) Activity

For ascorbate peroxidase (EC 1.11.1.1) activity 1g leaf tissue was homogenized in 6 ml of 100mM sodium phosphate buffer (pH 7.0) containing 5mM ascorbate, 10% glycerol and 1mM EDTA. Specific ascorbate peroxidase activity was assayed according to **Nakano and Asada (1981)**. The assay mixture consisted of 50 µL of the enzyme extract, 50 mM phosphate buffer (pH 6.0), 0.1 µM EDTA, 0.5 mM ascorbate, and 1.0 mM H_2O_2 in a total volume of 1.5 ml. Ascorbate oxidation was monitored by reading the absorbance at 290 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (ΔA_{290}) was divided by the ascorbate molar extinction coefficient (2.8 mM-1.cm-1) and the enzyme activity expressed as µmol of H_2O_2 $min^{-1}.mg^{-1}$ protein, taking into consideration that 1.0 mol of ascorbate is required for the reduction of 1.0 mol of H_2O_2 **(McKersie and Leshem, 1994).**

3.3.5.1.2.1.2 Guaiacol Peroxidase (GPX) Activity

Guaiacol peroxidase (GPX) (EC 1.11.1.7) activity was determined as described by **Urbanek *et al.* (1991).** 1.0g of leaf material was homogenized in 3.0ml of extraction buffer (100mM Phosphate buffer [pH:7.0] containing 0.1mM EDTA) in a pre-chilled mortar and pestle using liquid nitrogen. The homogenate was centrifuged at 16,000×*g* for 20 min at 4˚C. The supernatant fraction was used as crude enzyme extract for determination of enzyme activity. Total reaction mixture volume was 2ml, prepared in quartz cuvette. In 1850µl of assay buffer containing, 100mM phosphate buffer (pH 7.0), 0.1µM EDTA 50µl of enzyme extract was added. Reaction was started by adding 50µl of guaiacol (5 mM) and 50µl of H_2O_2 (15 mM) and the increase in absorbance was recorded at 470nm for 3min. The formation of tetraguaiacol was quantified using its molar extinction coefficient (26.6 $mM^{-1}cm^{-1}$). The enzyme activity expressed as $\mu molmin^{-1}mg^{-1}$protein.

3.3.5.1.2.1.3 Catalase (CAT) Activity

Leaves were homogenized in 100mM sodium phosphate buffer (pH 7.0) containing 1mM EDTA under liquid nitrogen. Specific Catalase (EC 1.11.1.6) activity was measured according to **Beers and Sizer (1952)**, with minor modifications. The assay mixture consisted of 50 µL of the enzyme extract, 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, and 20 mM H_2O_2 in a total volume of 1.5 ml. The decrease of H_2O_2 was monitored by reading the absorbance at 240 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance (ΔA_{240}) was divided by the H_2O_2 molar extinction coefficient (36 $M^{-1}.cm^{-1}$) and the enzyme activity expressed as µmol of H_2O_2 $min^{-1}.mg^{-1}$ protein.

3.3.5.1.2.2 Total Peroxidase (POD) Activity

The procedure of **Siegel and Galaston (1967)** was followed for Total Peroxidase Activity. 0.5 gm of leaf material was homogenized in 5 ml of extraction buffer (1 M Tris buffer (pH 7.8) with 2 % PVP), then centrifuged at 14,000xg for 20 minutes at 4° C and supernatant was collected. 200µl supernatant was mixed with 800 µl of assay buffer containing 0.2M Potassium phosphate buffer (pH 7.8) and 5mM Guaiacol. The reaction was started by adding 10ul of 5mM H_2O_2 and the change in absorbance was measured at 470nm after every 5 second.

The measured activity of total peroxidase was expressed in µmol min^{-1} mg protein $^{-1}$ using the extinction coefficient as 6.39 mM^{-1} cm^{-1} at 470nm.

3.3.5.1.3 Protein estimation by Bradford method:

The method based on Bradford (1976) was used for protein estimation.

3.3.5.1.3.1 Bradford dye

Dye solution:

Comassie brilliant blue G-250	10 mg
Absolute alcohol	5 ml
Orthophosphoric acid 85 %	10 ml

The volume was made up to 100 ml with distilled water. After dissolving the salt, resulting solution was filtered through Whattmann filter paper No. 2, in dark. The dye was stored in an amber colored bottle at 4^0C.

3.3.5.1.3.2 BSA standard

Stock solution : 1.0 mg/ml

1. Glass test tubes having 5, 10, 20, 40, 60, 100 µl of BSA in duplicate, were prepared. Extraction buffer was added to make up the final volume to 300µl.

BSA stock solution	Extraction buffer/ double distilled water	Total Volume (µl)
5µl	295µl	300µl
10µl	290µl	300µl
20µl	280µl	300µl
40µl	260µl	300µl
60µl	240µl	300µl
100µl	200µl	300µl
Blank	300 µl	300µl
Protein sample 1(40 µl)	260µl	300µl

2. 3 ml of dye was added in each tube.
3. The reaction mixture was incubated at RT for 15 minutes, in dark.
4. Absorbance was recorded at 595 nm against a reagent blank for all samples.
5. Graph was plotted between the Absorbance Vs Amount of protein in (µg).
6. The amount of in the sample was determined by extrapolation from the standard curve of BSA.

3.4. Chemicals and Glasswares

3.4.1 Source of Chemicals

All the chemicals used in the present study were of analytical/molecular grade and were purchased from Bangalore Genei, Hi Media, Merck, SRL and/or S. D. Fine Chemicals. Cytosolic Ascorbate peroxidase (*apx1*) and neomycin phosphor-transferase II

(*nptII*) gene primers used in this study were custom synthesized through Bangalore Genei.

3.4.2 Glasswares and Equipment

All the glassware's used in present study were of Borosil Company and plastic wares such as disposable petriplates, microfuge tubes, PCR tubes, micro tips and tip boxes were purchased from Tarson and Hi Media. Micropipettes were from Accupipet, M/s Tarson etc.

Following equipments were used:

S.No.	Equipment	Source
1.	pH meter	: Genei, Bangalore
2.	Spectrophotometer	: Thermo-spectronic
3.	Electronic Balance	: Precisa
4.	PCR	: Eppendorf, Germany
5.	Waterbath	: Orbitek
6.	Incubator Shaker	: Orbitek
7.	Laminar air flow	: Cleanair, India
8.	Autoclave	: Sanco, India
9.	Microwave oven	: BPL, India
10.	Horizontal electrophoresis system	: Genei, Bangalore
11.	Deep freezer (-20^{0}C)	: Vest frost
12.	Gel Documentation system	: Alphainnotech
13.	Digital camera	: canon
14.	Centrifuge	: Eppendorf, Germany
15.	Hot Air Oven	: Sanco, India
16.	Fridge	: LG, India
17.	IRGA	: CID Inc. USA
18.	SPAD	: Opti Science, USA
19.	PEA meter	: Hansatech, UK

Results and Discussion

Soil salinity is a major constraint limiting agricultural productivity on nearly 20 percent of the cultivated area and half of the irrigated area worldwide **(Zhu, 2001)**. Salt stress has been reported to cause an inhibition in growth and development through retardation of fundamental metabolic processes like photosynthesis, respiration & protein synthesis.

Soil and water salinity is caused by the presence of excessive amounts of salts and their constituent ions. Most commonly, Na^+ and Cl^- cause salinity stress. The toxic effect of salt stress manifest in three different ways: 1) it reduces water potential, 2) causes ion imbalance or disturbances in ion homeostasis and 3) leads to the generation of excessive ROS. The altered water status leads to initial growth reduction and greatly limits plant productivity. Since salt stress involves both osmotic and ionic stress (**Hagemann and Erdmann, 1997; Hayashi and Murata, 1998)**, growth suppression is directly related to total concentration of soluble salts or osmotic potential of soil water **(Flowers *et al.*, 1977; Greenway and Munns, 1980)**. The disturbances in water status and ion homeostasis cause perturbations in a wide variety of metabolic activities. Formation of reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) **(Halliwell and Gutteridge, 1985)**, and singlet oxygen $^1O_2$) **(Elstner, 1987)** are the secondary effects of salinity stress that results from altered cellular metabolism. These cytotoxic activated oxygen species can seriously disrupt cellular structure and function through oxidative damage to lipids **(Fridovich, 1986; Wise and Naylor, 1987)** proteins and nucleic acids **(Fridovich, 1986; Imlay and Linn, 1988)**.

Plants have developed several mechanisms to defend themselves against reactive oxygen species generated under environmental stresses including salinity stress **(Foyer *et al.*, 1994; Inze and Van Montagu, 1995)**. The antioxidant defense system of plants include antioxidant molecules such as ascorbate, gluatathione and α–tocopherol which are directly responsible for detoxification of ROS **(Alscher *et al.*, 1997)** and enzymes like ascorbate peroxidase (APX) superoxide dismutase (SOD), glutathione reductase (GR) and Monodehydro ascorbate reductase (MDHAR) **(Foyer *et al.*, 1994; Allen *et al.*, 1995)**; which show an increased activity, as a result of exposure to stress. One of the key enzymes for detoxifying ROS in plant cells is Ascorbate peroxidase which detoxifies H_2O_2 to $H_2O + O_2$ **(Asada, 1992)**. APX is one of the most widely distributed antioxidant enzymes in plant cells **(Wang *et al.*, 1999)**.

APX is a part of ascorbate-glutathione cycle, which uses ascorbate to reduce H_2O_2 **(Foyer and Halliwell, 1976)**. The cycle is the most important H_2O_2 detoxifying system in the chloroplast and has also been identified in the cytosol, peroxisomes and mitochondria **(Nakano and Asada, 1981)**. Since the cytosolic APX cDNAs were the first to be isolated, this enzyme has so far received the maximum attention.

However, H_2O_2 being easily diffusible through biological membranes, accumulate excessively in the cytosol. Incidentally, Cytosolic isoform of APX is the one which responds maximally to externally imposed stress **(Fourcroy *et al.* 2004; Davletova *et al.* 2005).**

Therefore in the present investigations an attempt was made to genetically engineer *Brassica juncea* (*var.* pusa jaikisan) for over-expressing one of the key regulatory enzymes of the plant antioxidant defense pathway i.e. cytosolic ascorbate peroxidase so as to

strengthen the antioxidative defense system of the affected plant. It was hypothesized that the transgenic plants with an ectopically enhanced activity of ascorbate peroxidase will have an increased tolerance to oxidative stress.

The results obtained during the course of investigation have been divided and described in following three parts.

1. The first part deals with optimization of regeneration protocol from hypocotyl explants of *Brassica juncea* (*var.* pusa jaikisan).
2. Second part deals with the development of transgenic plants through *Agrobacterium* mediated genetic transformation of *Brassica juncea* with *apx1* gene and their physiological and molecular analysis.
3. The third part deals with the evaluation of oxidative stress tolerance potential of transgenic plants exposed to salinity stress; using standardized biochemical parameters.

4.1 Regeneration Studies on *Brassica juncea* var. pusa jaikisan

4.1.1 *In vitro* plant regeneration

Brassica species have been widely exploited for tissue culture purposes and regeneration protocols have been developed for most of the species. However, optimization of regeneration protocols before attempting genetic transformation is essential because no two organisms have identical genomes. Direct organogenesis has been the widely accepted pathway for regeneration & transformation in *Brassica* crops compared to other methods of regeneration like somatic embryogenesis.

One of the essential requirements for an efficient transformation system in a plant species is the availability of a reproducible and efficient regeneration protocol. In the present investigations plant regeneration was done via organogenesis *i.e.*, the formation and

outgrowth of monopolar organs e.g. shoots and roots directly from the explants. These organs develop procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the explants **(Flick *et al.*, 1983)**. Plant regeneration via organogenesis can be achieved either through production of organs from a callus mass derived from the explant or through the emergence of adventitious organs directly from the cut ends of the explants, without an intervening callus phase. Regeneration of plants via organogenesis has been accomplished from various tissues such as cotyledons **(Sharma *et al.*, 1990),** hypocotyl segments **(Eapen and George, 1997),** leaves **(Radke *et al.*, 1988),** thin cell layers of epidermal and sub epidermal cells **(Klimaszewska and Keller, 1985),** roots and protoplasts **(Glimelius, 1984; Hu *et al.*, 1999).**

Hypocotyl segments were used in the present experiments, as they are the most frequently used explants for Brassica transformation, which are easy to establish under tissue culture and have been reported to yield higher transformation efficiency **(Barfield and Pua, 1991)**. **Glimelius (1984)** has also previously reported that hypocotyls possess high morphogenetic potential and are an excellent starting material for plant regeneration. However, certain other reported transformation systems also use cotyledonary petiole as explants **(Moloney et al., 1989)**. Therefore, as a first step towards transformation, regeneration from hypocotyls of Brassica juncea var. pusa jaikisan was optimized.

4.1.2 Effect of plant growth regulator combinations on Shoot Regeneration

Specific growth regulator concentrations in the culture medium are critical for optimal morphogenetic response **(Chawla, 2002).** Auxin and cytokinins concentration have been known to profoundly

influence callus induction and shoot regeneration. Varying their concentrations in the culture medium, cause difference in shoot/callus induction response and growth pattern of explants. α-Naphthalene acetic acid (NAA) has been reported to be superior to other auxins **(Klimaszewska and Keller, 1985; Narasimhulu and Chopra, 1988)** for eliciting organogenesis response in *Brassica* explant cultures. The most frequently used cytokinin in Indian mustard tissue culture is 6-benzyl amino purine (BAP) **(Barfield and Pua, 1991; Dhawan *et al.*, 2004)**. 6-benzyl amino purine has typically been used in excess as compared to NAA.

For standardizing the best combination of NAA and BAP for regeneration in *Brassica juncea*; hypocotyls, 0.8 to 1.0 cm long and obtained from 6-day-old seedlings of *Brassica juncea var.* pusa jaikisan were aseptically cut and transferred to MS basal medium **(Murashige and Skoog, 1962)** supplemented with different concentrations of NAA (0.5, 1.0, 1.5 2.0 & 2.5 mg/L) and BAP (0.5, 1.0, 1.5 2.0 & 2.5 mg/L) in a graded fashion. Explants were cultured in petriplates and all the treatments were performed with 5 replication events. Data on per cent callus induction frequency and per cent shoot induction frequencies (% CIF and % SIF) were recorded.

After one week of culture, the hypocotyl explants show swelling that starts from the ends, followed by callus induction or direct shoot initiation within three weeks of culture (Figure 4.1, 4.2). Callus formation was also initiated from the cut ends. Greenish yellow callus formation was observed in almost all the combinations of NAA and BAP used, although with different frequencies. Callus formation and shoot regeneration response in different treatments was observed after 4-5 weeks of culture (Table 4.1).

Table 4.1: Callus and Shoot induction frequency of hypocotyl explants obtained from 6-day-old *B. juncea* seedlings and cultured on MS medium supplemented with different concentrations of BAP (B) and NAA (N)

Conc. of NAA & BAP (mg/L)	No. of explants	No. of explants producing callus	% Callus Induction Frequency	No. of explants producing shoots	% Shoot Induction Frequency
0B0N	15	0	0.00	0	0.00
0.5B 0.5N	15	10	66.67	4	26.67
0.5B 1N	15	9	60.00	6	40.00
0.5B 1.5N	15	10	66.67	5	33.33
0.5B 2N	15	12	80.00	6	40.00
0.5B 2.5N	15	11	73.33	7	46.67
1B 0.5N	15	13	86.67	8	53.33
1B 1N	15	15	100.00	8	53.33
1B 1.5N	15	7	46.67	5	33.33
1B 2N	15	7	46.67	7	46.67
1B 2.5N	15	8	53.33	4	26.67
1.5B 0.5N	15	9	60.00	5	33.33
1.5B 1N	15	11	73.33	7	46.67
1.5B 1.5N	15	10	66.67	5	33.33
1.5B 2N	15	9	60.00	6	40.00
1.5B 2.5N	15	8	53.33	4	26.67
2B 0.5N	15	10	66.67	7	46.67
2B 1N	15	10	66.67	4	26.67
2B 1.5N	15	7	46.67	7	46.67
2B 2N	15	9	60.00	6	40.00
2B 2.5N	15	8	53.33	8	53.33
2.5B 0.5N	15	10	66.67	10	66.67
2.5B 1N	15	11	73.33	13	86.67
2.5B 1.5N	15	8	53.33	11	73.33
2.5B 2N	15	7	46.67	9	60.00
2.5B 2.5N	15	9	60.00	8	53.33
S.Em.±	–	–	1.10	–	1.32
CD at 5%	–	–	3.14	–	5.89
CV (%)	–	–	3.14	–	4.4

Amongst all the plant growth regulator combinations, variety pusa jaikisan showed maximum 86.67 per cent shoot induction frequency on medium supplemented with 2.5 mg/L BAP and 1.0 mg/L NAA. The per cent callus induction frequency was found maximal (100%) on medium having 1 mg/L BAP & 1 mg/L NAA. Therefore 2.5 mg/L BAP and 1.0 mg/L NAA combination was selectively used for shoot regeneration from hypocotyl explants of *Brassica juncea* in all further experiments.

From comparison of the results obtained on media supplemented with 2.5 mg/L BAP & 0.5 mg/L NAA (66.67% SIF), 2.5 mg/L BAP & 1.0 mg/L NAA (86.67% SIF) and 2.5 mg/L BAP & 1.5 mg/L NAA (73.33% SIF), it is clear that the presence of 2.5 mg/L BAP in the growth medium essentially increases the organogenic response. A supplementation of 1.0 mg/L BAP & 1.0 mg/L NAA, favored callus induction while higher concentrations favor shoot induction response. In general lower concentrations of BAP favor callus induction. These results indicate that organogenesis in *Brassica juncea* depends on the ratio as well as the absolute concentration of BAP and NAA in the growth medium.

4.1.3 Mode of shoot regeneration from hypocotyls

In case of hypocotyl derived shoot regeneration in *Brassica juncea*; it was found that leaves appeared at the initial stage of shoot regeneration before the complete shoots could be formed; suggesting that shoots were regenerated through organogenesis. Many “green centers” were observed on hypocotyl-derived greenish yellow callus after 20-25 days of culture (Figure 4.1 C). Shoots were found to emerge from the green centers of the hypocotyl derived callus (Figure 4.1 D), after 20 days of culture.

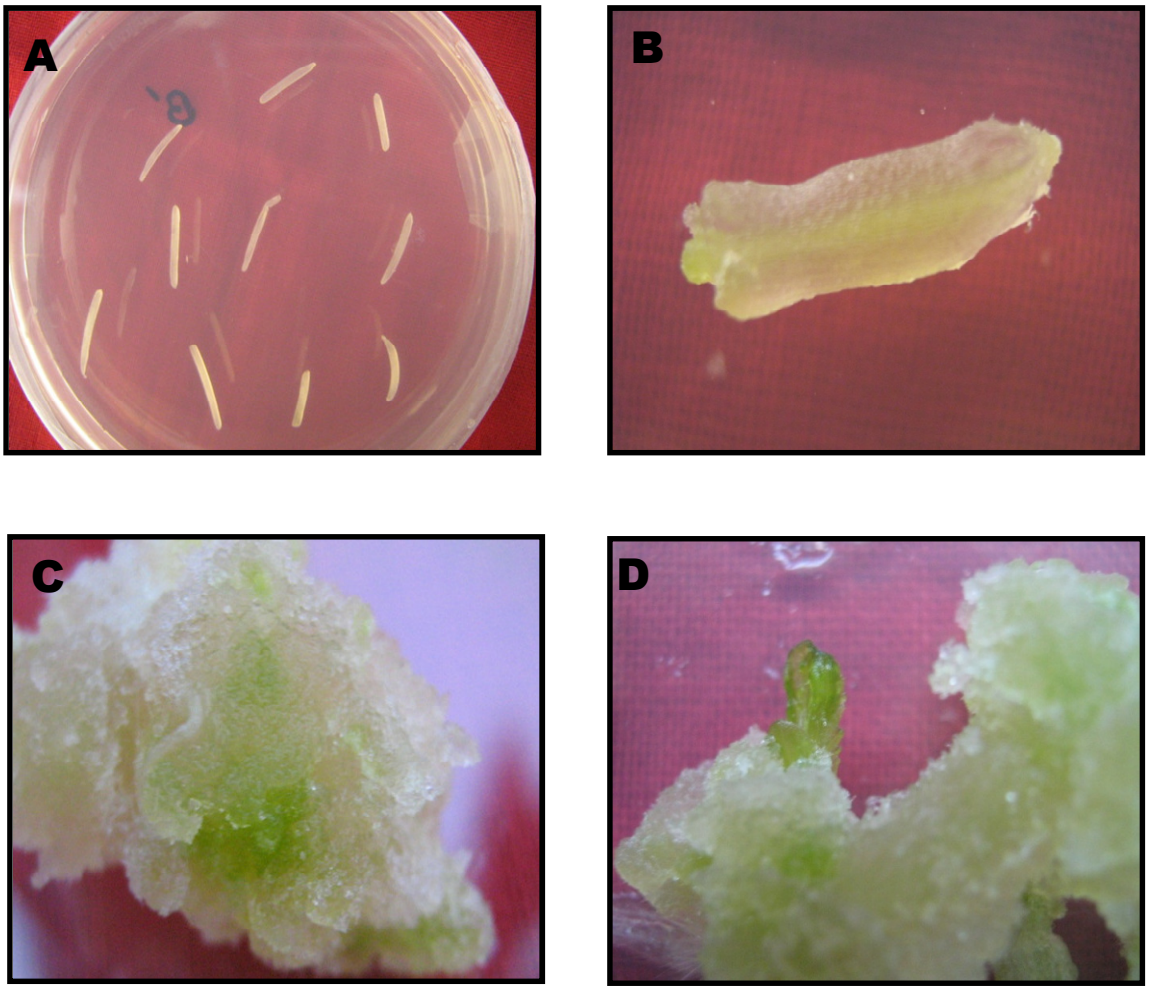


Figure 4.1: Callus and shoot induction response

A. Hypocotyl Explants

B. Explant after 7 days of culture

C. Organogenic Callus

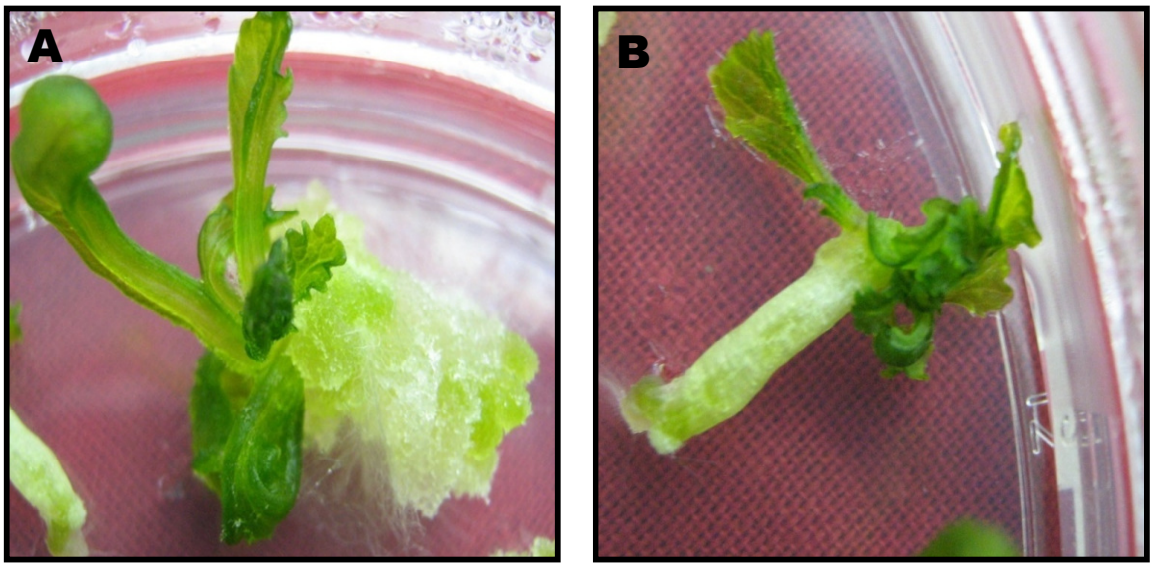
D. Emergence of shoot bud from callus

4.1.4 Multiple Shoot Induction

The shoots regenerated in shoot regeneration medium (SR_1) were selected for further multiple shoot Induction experiments. The regenerated shoots were given sharp cut and transferred in multiple shoot Induction media containing MS basal media supplemented with 2.5mg/L BAP alone and 3.4 mg/L $AgNO_3$. A number of new shoots emerged from the nodes of the regenerated shoots, placed on the multiple shoot Induction media (Figure 4.2 C). **Prasad *et al.* (2000)** have also successfully used 2.5 mg/L BAP for shoot regeneration in *Brassica*. In our experiments it was noticed that presence of $AgNO_3$ in the growth medium enhances the regeneration potential. Different laboratories working on shoot regeneration of *Brassica* have also reported the use of $AgNO_3$ to be obligatory for enhancing shoot regeneration from different explants **(Barfield and Pua, 1991; Mukhopadhyay *et al.*, 1992; Xiang *et al.*, 2000).** $AgNO_3$ dissociates to provide silver ions, which are potential inhibitors of ethylene biosynthesis. Ethylene could possibly inhibit or retard the *de novo* organogenesis during tissue culture **(Barfield and Pua, 1991)**. Further silver ions are known to inhibit the synthesis of phenolic compounds, which may further enhance the regeneration potential from different explants.

4.1.5 Root induction from regenerated shoots

Young differentiated *Brassica* shoots approximately 2-3 cm in height were cut and transferred to MS medium supplemented with different concentrations (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) of Indole-3 butyric acid(IBA) for inducing root formation (Table 4.2). Care was taken to make a sharp and oblique cut on the shoots, as blunt cut on the shoot did not allow rooting to take place. Cut end of the shoot was immersed in semi-solid root induction medium. Root induction in



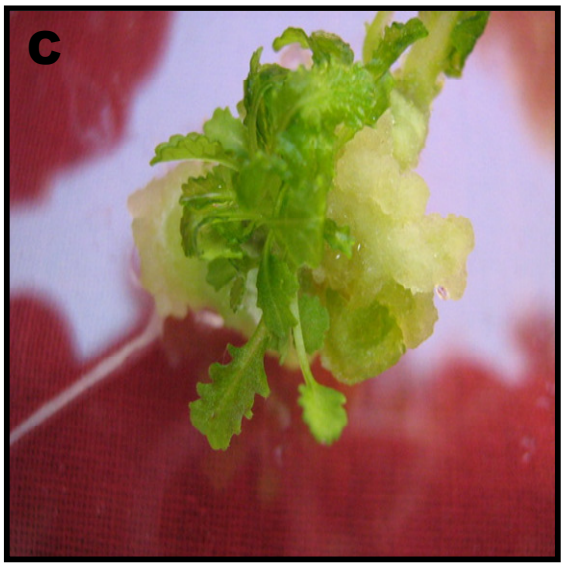


Figure 4.2: Shoot induction response

A. **Initiation of shoot from callus**

B. **Direct shoot induction from hypocotyl explants**

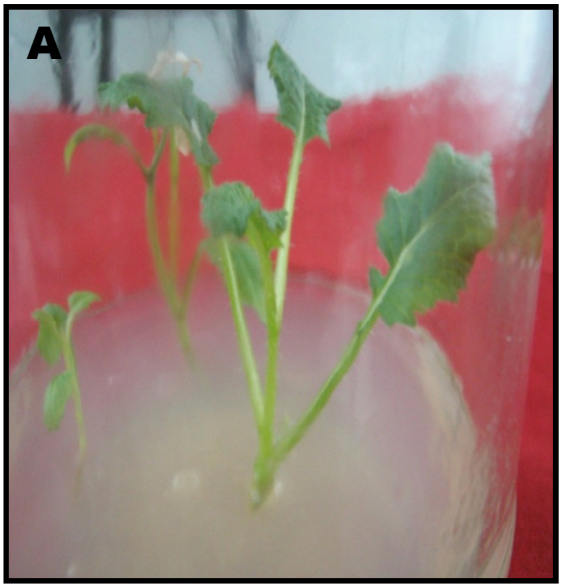
C. **Multiple shoot induction through callus**

different treatments started within a period of 7 to 10 days and rooting became much more profuse after three weeks of culture (Figure 4.3).

The per cent root induction frequency (section 3.3.1.7) ranged from 30 to 90% in different treatments of IBA. The medium with 0.5 mg/L IBA showed maximum root induction frequency of 90% after 3 weeks of culture (Table 4.2). It was observed that with an increase in the concentration of IBA in the rooting medium, the root induction frequency went down consistently. Therefore 0.5 mg/L IBA was selected for rooting of differentiated *Brassica* shoots, in subsequent experiments of regeneration and transformation. Several scientists have reported profuse rooting response of regenerated *Brassica* shoots on MS medium supplemented with IBA **(Basu *et al.*, 2001 and Sharma *et al.*, 2004)**.

Table 4.2: Effect of different concentrations of IBA on per cent root induction response of differentiated shoots in full length MS medium

IBA (mg/L)	Number of shoots placed in rooting medium	Number of shoots giving rooting response	Percentage root induction
0	10	6	60
0.5	10	9	90
1.0	10	7	70
1.5	10	4	40
2.0	10	5	50
2.5	10	3	30
S.Em.±	–	-	0.83
CD at 5%	–	-	2.58
CV (%)	–	-	2.56



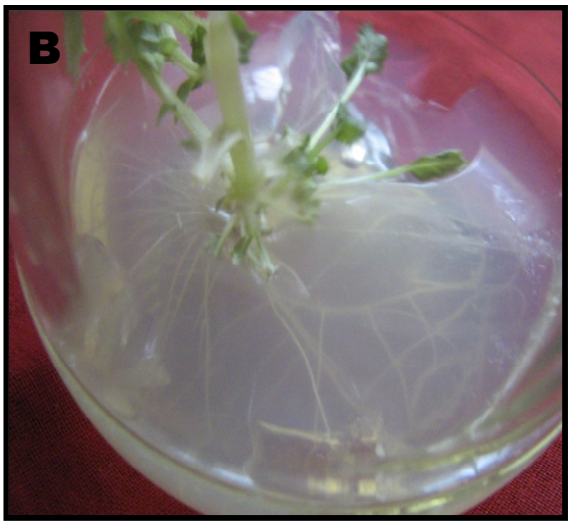


Figure 4.3: Root induction from differentiated shoots

A **Regenerated shoots of *Brassica juncea* placed in root induction medium.**

B **Rooting of regenerated shoots**

4.1.6 Hardening of Plantlets

The *in vitro* rooted plantlets were taken out from the culture vessels and their roots were washed with running tap water so as to remove pieces of agar. The clean plantlets were transferred to disposable plastic cups filled with sterilized coco-peat. They were immediately watered and kept covered with perforated poly-bags in temperature controlled glass house under 70%-80% humidity. After 12 to 15 days the poly-bags were removed and the plantlets were grown for another 4 weeks under similar conditions. The plants were watered at regular intervals. Young plants were transferred to plantation pots and grown up to maturity. The seeds from mature *Brassica* plants were collected and stored in a desiccator.

4.2 TRANSFORMATION OF *Brassica juncea* (var. pusa jaikisan) WITH *Agrobacterium tumefaciens* STRAIN C58C1RIF^R pGV2260 HARBORING ptpApx

4.2.1 Confirmation of *Agrobacterium tumefaciens* strain C58C1RIF^R pGV2260:

The *Agrobacterium* strain C58C1rif^R was inoculated in liquid YEB media containing 100mg/L rifampicin, 100mg/L spectinomycin and 100mg/L spectromycin. Pure culture was streaked on plates containing fresh semi-solid YEB media and used for further experiments.

Growth curve of *Agrobacterium tumefaciens* was prepared (Figure 4.4); the results show that optimal density of *Agrobacterium* in the log phase is achieved after 12-14 hours of inoculation. Therefore, *Agrobacterium* cells for all further experiments were harvested after 14 hours of inoculation.

The plasmid ptpApx was isolated from 50 ml of *Agrobacterium* culture and analyzed on 0.8 % agarose gel. The results of plasmid isolation are shown in Figure 4.5 the desired size (12.09kb) of plasmid DNA band was obtained in the analysis.

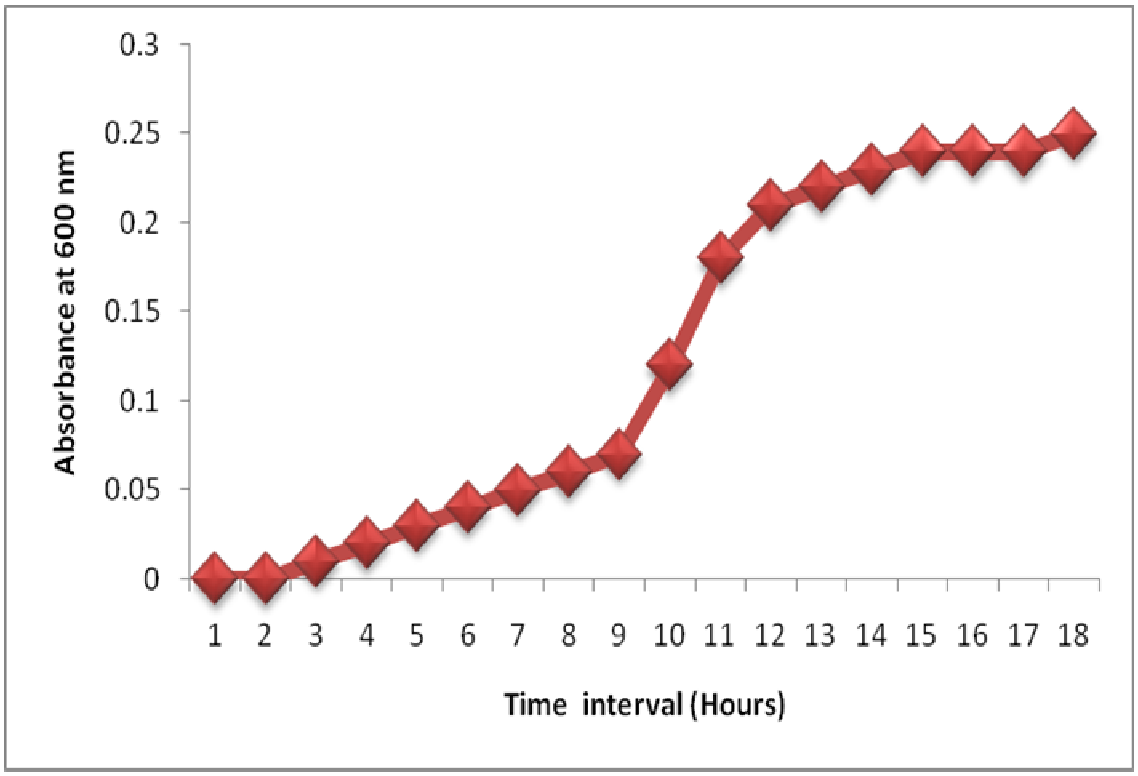


Fig. 4.4: Growth curve of *Agrobacterium tumefaciens*

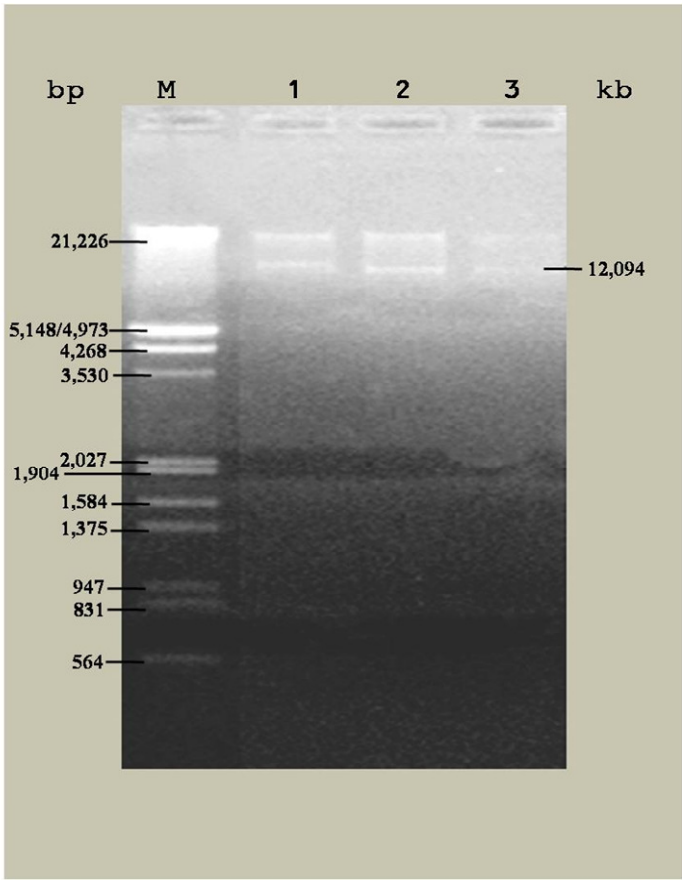


Figure 4.5: ptpApx Plasmid DNA isolated from *Agrobacterium tumefaciens* Strain C58C1rif^{R} pGV2260
Lane M: Molecular weight marker
Lane 1-3: Plasmid ptpApx

4.2.2 Confirmation of *apx1* gene insert in plasmid ptpApx through colony PCR

The presence of *apx1* cDNA construct in the *Agrobacterium* strain was confirmed through colony PCR using gene specific primers for Cytosolic ascorbate peroxidase (*apx1*) and neomycin phosphotransferase (*nptII*). The PCR amplification by *apx1* and *nptII* specific primers gave a product size of 898bp and 734bp respectively (Figure 4.6). This matches with the expected product size as calculated through online primer designing program (primer3). This reaction was used as a positive control in further PCR experiments.

4.2.3 Studies on *Agrobacterium* Mediated Transformation

Among the various methods used for genetic transformation for crop plants, *Agrobacterium* mediated transformation is most commonly used, as it offers many unique advantages such as (i) simple methodology (ii) Transfer and integration in host genome with defined ends (iii) Co-transformation of selectable marker along with the gene of interest (iv) low incidence of gene silencing (v) ability to transfer reasonably long stretches of DNA (approx. 150 kb).

The genetic transformation experiments were carried out to develop and test transgenic *Brassica juncea* plants against salinity stress, using *apx1* cDNA in the vector ptpApx carried by the *Agrobacterium* strain C58C1rifR pGV2260.

During the process of *Agrobacterium* mediated transformation, T-DNA is mobilized from *Agrobacterium* to plant cell where it gets integrated in the host plant's genome in one or multiple copies. T-DNA integration makes the recipient plant cell resistant to Kanamycin and also ensures that the target gene i.e. *apx1* is also integrated. Therefore, in order to determine the Kanamycin concentration, which could be used to select transformed hypocotyls, Kanamycin sensitivity test of hypocotyls was carried out.

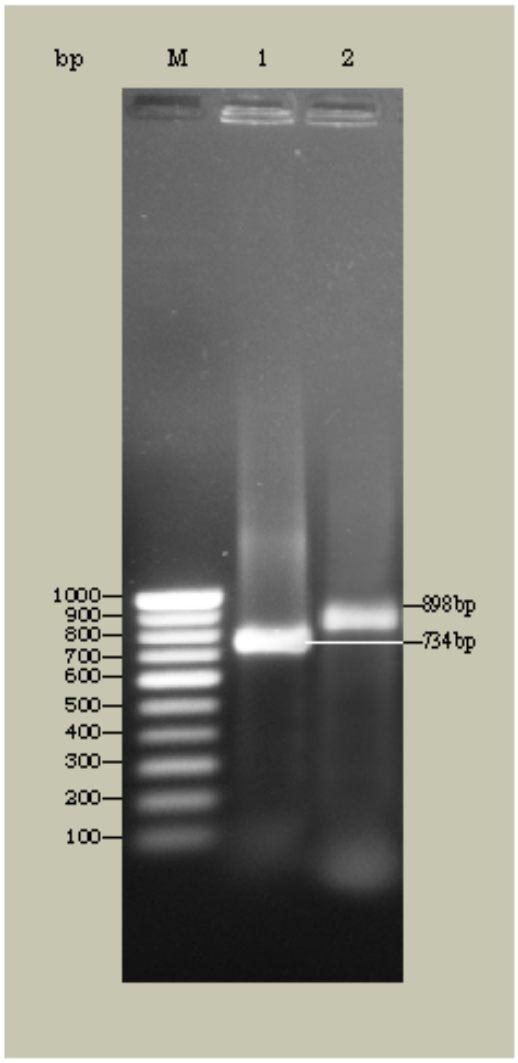


Figure 4.6: PCR analysis of ptpApx plasmid.
Lane [M]: Molecular weight marker
Lane [1]: Colony PCR with *nptII* primer
Lane [2]: Colony PCR with *apx1* primer

4.2.3.1 Kanamycin sensitivity test

The antibiotic serves as a selective agent that allows only transformed cells, tissues or plantlets to survive. Kanamycin has been extensively used as a selective antibiotic in transformation experiments **(Bevan *et al.*, 1983)**.

Kanamycin inhibits the growth of plant cells by preventing initiation and translation at 30S ribosomal subunits. Virtually, a transformed cell, which carries Kanamycin resistant gene, should be able to grow normally and divide in presence of Kanamycin, while the untransformed cells will not be able to grow and multiply.

Therefore, to find out the appropriate concentration of Kanamycin, which could effectively kill untransformed cells, kanamycin treatment of untransformed explants was carried out by culturing explants on MS growth media supplemented with increasing concentrations of kanamycin (0, 10, 20, 30, 40 and 50 mg/L). Data recorded after three weeks of culture (Table 4.3) shows that even 10 mg/L of Kanamycin could retard callus growth and development. However, 30 mg/L of Kanamycin treatment totally inhibited growth of untransformed explants (Figure 4.7). At 30 mg/L of Kanamycin, all the untransformed explants turned necrotic and died in less than one week. Therefore, 30 mg/L of kanamycin was used to finally select the transformed cells/tissues in sequential transformation experiments.

Table 4.3: Kanamycin sensitivity test of hypocotyl explants obtained from 6 day old seedlings of *B. juncea* (*var.* pusa jaikisan)

Media	Kanamycin concentration(mg/L)	No. of cultured Explants	% of surviving explants
MSG0	0	30	100
MSG1	10	30	6
MSG2	20	30	2
MSG3	30	30	0
MSG4	40	30	0
MSG5	50	30	0

Figure 4.7: Untransformed shoots transferred on MS growth media supplemented with 30 mg/l of kanamycin.

4.2.3.2 Transformation of *Brassica juncea* (*var.* pusa jaikisan) using *Agrobacterium* strain C58C1rif^R pGV2260

Hypocotyl segments, obtained from 6-day old *B. juncea* seedlings, were used as starting material in *Agrobacterium* mediated transformation. Different steps, followed during *Agrobacterium* mediated transformation of hypocotyls and the observations made are as follows:

Pre-culture: Fresh hypocotyl explants were pre-cultured in liquid PCM medium for 24-48 hours with constant shaking (100 rpm) at 28^{0}C under diffuse light. On the basis of transformation frequency obtained with varying duration of pre-culture treatment, 48 hours of pre-culture treatment was finalized for obtaining maximal transformation efficiency.

Infection: The hypocotyl explants were suspended in the *Agrobacterium* suspension (Abs = 0.2-0.3) for 30 min under sterile conditions with constant shaking (100 rpm) at 28^{o}C.

Co-cultivation: After infection, explants were allowed to co-cultivate for 24 hours at 28^{0}C @ 110 rpm by placing them in liquid regeneration medium (PCM). This step is of critical importance as the T-DNA transfer from *Agrobacterium* to the host cell occurs during this time.

Washing: After Co cultivation, hypocotyl explants were washed thrice for 30 minutes each with WM (Autoclaved double distilled water + 300 mg/L cefotaxime) with continues shaking.

Plating of co-cultured hypocotyl explants: The co-cultured hypocotyl explants, after washing were plated, on regeneration medium (SR1) containing 30 mg/L kanamycin and 300 mg/L cefotaxime, for shoot regeneration (Figure 4.8). However, it was noticed that the hypocotyl segments turned necrotic after few days of

Figure 4.8: Shoot regeneration from kanamycin resistant callus derived from putatively transformed hypocotyl explants

A) Callus induction from the cut ends of putatively transformed hypocotyl explants

B) Shoot initiation from callus (in a closer view)

co-cultivation with *Agrobacterium*. After 12 to 15 days of culture on shoot induction media with kanamycin, the hypocotyl segments turned brown and died soon. To avoid this the acclimatization time in the pre culture media was increased to 48 hours. Preculture of hypocotyls explants has been reported to be an essential step in transformation by several workers **(Babic *et al.*, 1998; Xiang *et al.*, 2000).** This step is also proposed to induce competence in hypocotyl segments for *Agrobacterium* transformation, besides lowering the stress. Secondly, a two step cycle was adopted for selection of transformed explants. Initially, after co-cultivation and washing the transformed explants were selected on 20 mg/L of kanamycin and once the transformed tissue started growing, the selection pressure was increased to 30 mg/L. These changes resulted in better survival rate of the explants (Figure 4.9). The improvement in transformation efficiency can possibly be attributed to the initiation of active cell division upon wounding and improved infection ability of *Agrobacterium* to the newly synthesized cell wall at the wounding sites **(Sangwan *et al.*, 1992)**.

During the process of selection, transformed hypocotyls continued to grow vigorously to produce calli, whereas the untransformed ones failed to form callus and eventually got bleached and became necrotic within 2 to 3 weeks of culture. The surviving explants after 3 weeks of culture were transferred to SR_3 selection medium containing 30 mg/L kanamycin (Figure 4.10 A).

The elongated shoots were excised from calluses and transferred to multiple shoot induction media containing MS constituents along with 2.5mg/L BAP, 30 mg/L kanamycin, 3% sucrose and 0.8% agar. It was observed that all the green shoots developed after selection in shoot induction media did not remain green and healthy; some turned white and purple whereas some remained green. **Barfield and Pua**

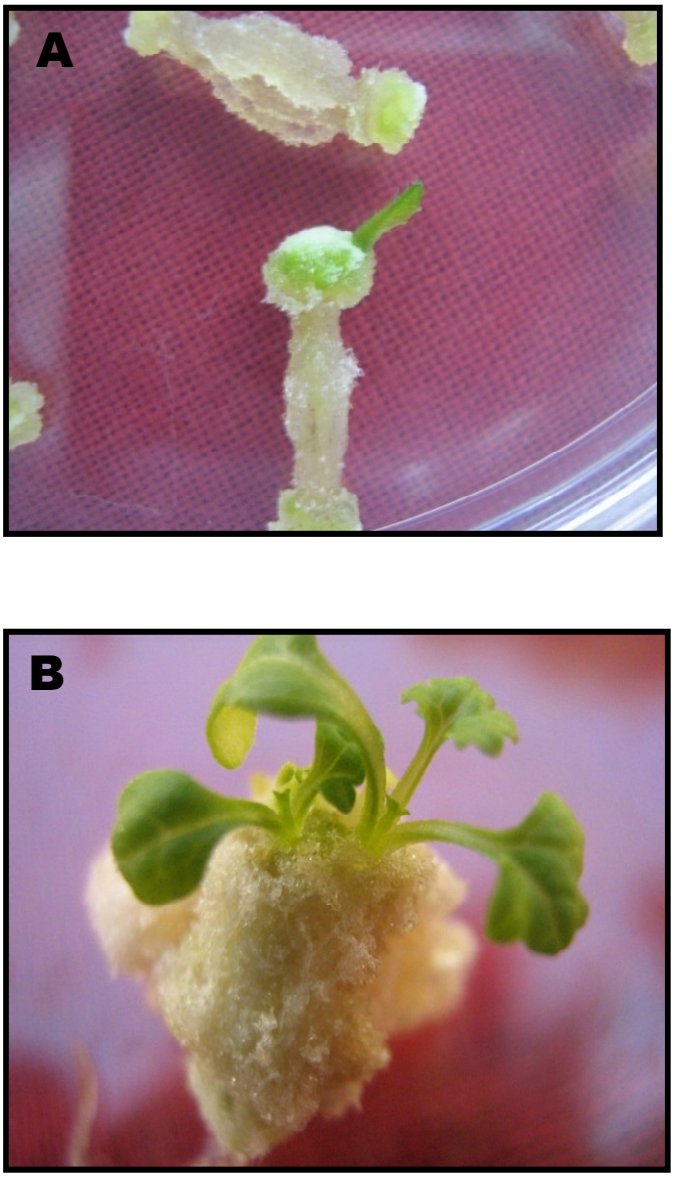


Figure 4.9: Formation of green shoot buds (A) and shoot regeneration (B) from green callus, cultured on shoot regeneration medium supplemented with 20 mg/L kanamycin

Figure 4.10: Shoot development from putatively transformed hypocotyls

A. Well developed kanamycin resistant shoots, after 12 weeks of culture

B. Kanamycin susceptible shoots, cultured on MS growth media supplemented with 30 mg/L kanamycin

(1991) have reported that non-transformed escapes occurred at reportedly high frequency of 68% while using kanamycin as a selectable marker.

After twelve weeks of selection and multiple shoot induction, healthy shoots of about 2 to 3 cm in height, were transferred to the root induction medium containing MS constituents along with 0.5 mg/L IBA, 20 mg/L Kanamycin, 3% sucrose and 0.8% agar for root initiation. The Kanamycin concentration was reduced to 20 mg/L, as it has been reported that root induction is sensitive to kanamycin as compared to shoot organogenesis and further explains the fact that the utility of any antibiotic depends on both, the plant species as well as the explant involved **(Saini *et al.*, 2003)**. Root induction started after one week of transfer and the shoots acquired a well developed root system after about three to four weeks of culture in root induction medium (Figure 4.11).

The transformed green and healthy rooted plantlets were transferred to disposable plastic cups containing coco-peat after proper washing away the agar pieces from root surface (Figure 4.12 A). Plantlets were initially covered with perforated plastic bags, for 7-10 days and kept in transgenic glass house under controlled temperature & humidity. After about four weeks, plants were transferred to plantation pots and grown up to maturity in the ambient environment of the transgenic glass house at 25 ^{0}C with 60-70 % humidity and natural photoperiod (Figure 4.12 B). At the time of flower initiation, the floral buds were covered plastic bags, to prevent cross pollination (Figure 4.12 C). The seeds obtained from mature transgenic plant were collected (Figure 4.12 D) and securely kept in a dessicator for further experiments.

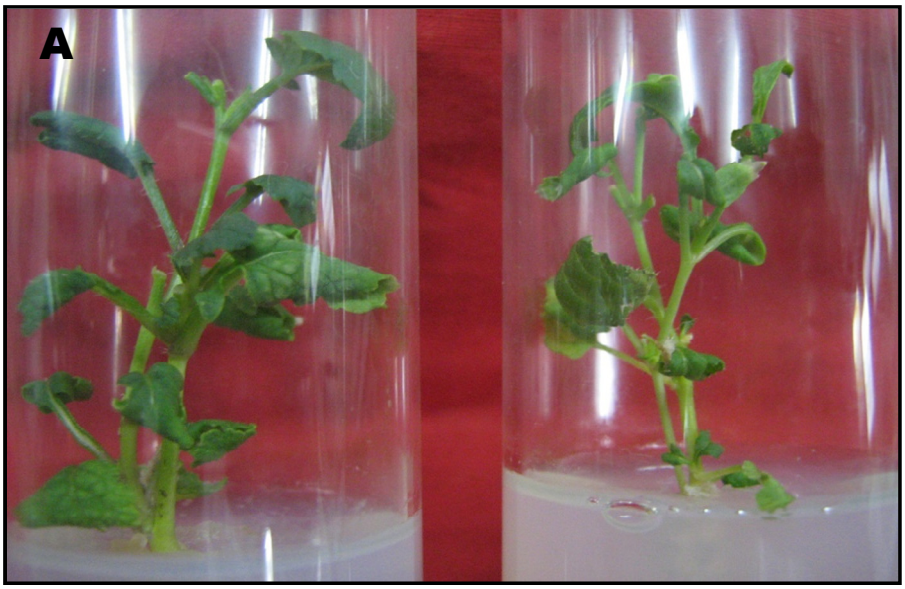


Figure 4.11: Root induction from putatively transformed shoots

A) Shoots in root induction media supplemented with 20mg/L kanamycin

B) A plantlet showing profuse rooting.

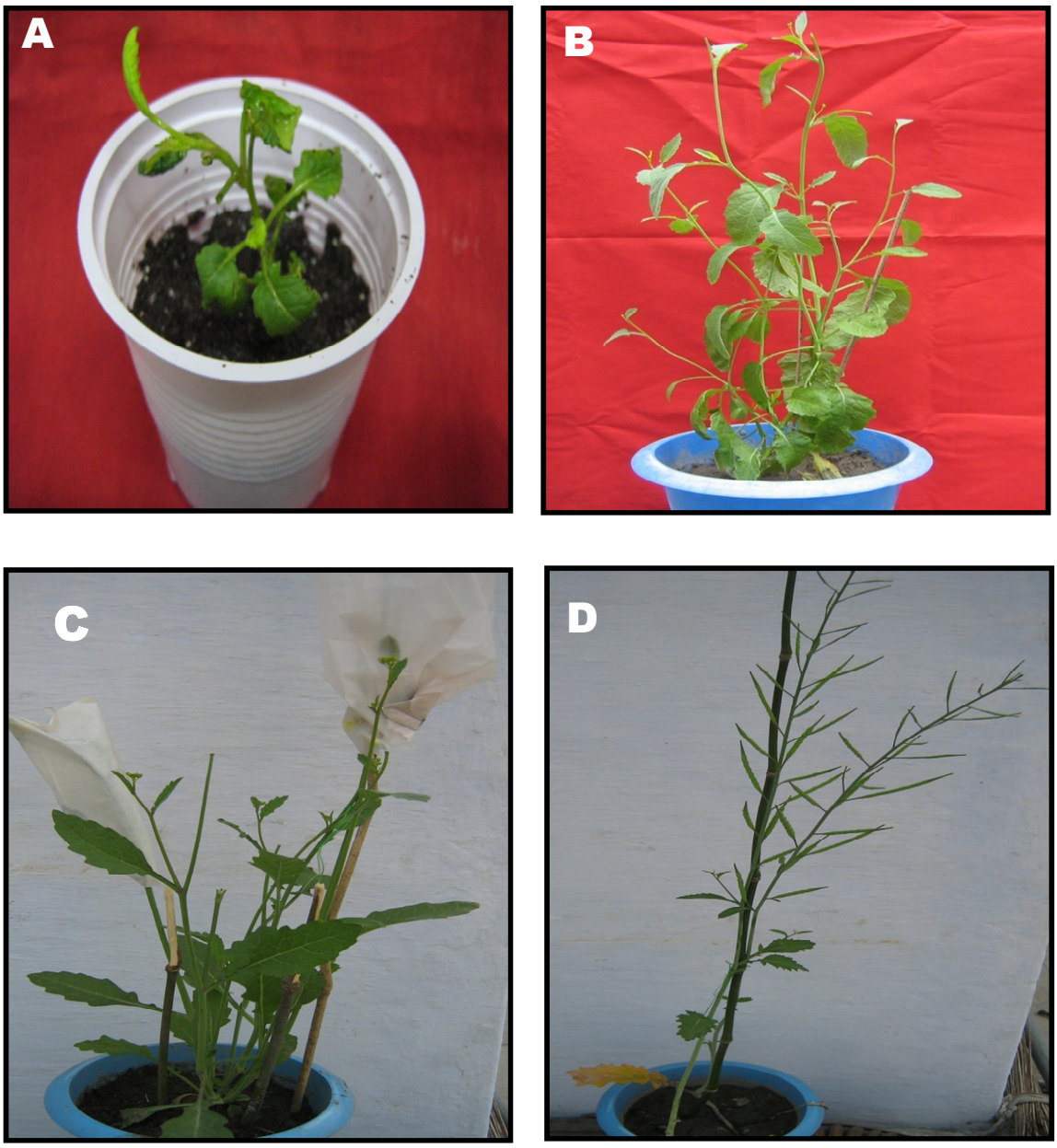


Fig. 4.12 Different stages of hardening and plantlet development

A. Transgenic plantlets in disposable plastic cups containing coco-peat.

B. Hardened transgenic plant growing in plantation pot.

C. Transgenic plant bearing flowers

D. Transgenic plants bearing pods.

4.2.3.3 Transformation Efficiency

Transformation efficiency is the ratio of kanamycin resistant plantlets regenerated to the initial (starting) number of hypocotyl explants treated with *Agrobacterium*. In a single experiment, out of a total of 600 *Agrobacterium* treated hypocotyl explants placed on kanamycin selection medium, seven (1.17%) healthy regenerated plantlets (T_0) were transferred to soil. The whole cycle was completed in approximately six months. However, all the *Brassica* plantlets that survived in the 30 mg/L Kanamycin selection pressure for consecutive cycles, tested PCR positive for the presence of insert. The frequency of regeneration of putative transformed plants from cultured explants and the frequency of transformed plants from among the putative transformed plants is typically reported to be rather low **(Moloney *et al.*, 1989).** Our results are in agreement with those reported by **Xiang *et al.*, (2000) and Moghaieb *et al.*, 2006.**

4.2.4 Confirmation of Transformation

4.2.4.1 Kanamycin Sensitivity Assay:

Neomycin phosphotransferase (*nptII*) expression was assayed on small leaf segments of approximately 1 cm^2 area, suspended in modified regeneration medium containing 30 mg/L Kanamycin. Leaf segments from wild type plants turned white within 3-5 days; while leaf segments from putative transgenic plant remained green (Figure 4.13), during this duration.

4.2.4.2 PCR confirmation of putative transgenic plants

4.2.4.2.1 Isolation, Purification and Quantification of Genomic DNA

The major prerequisite for PCR amplification of 'gene of interest' is a good quality genomic DNA preparation. DNA was

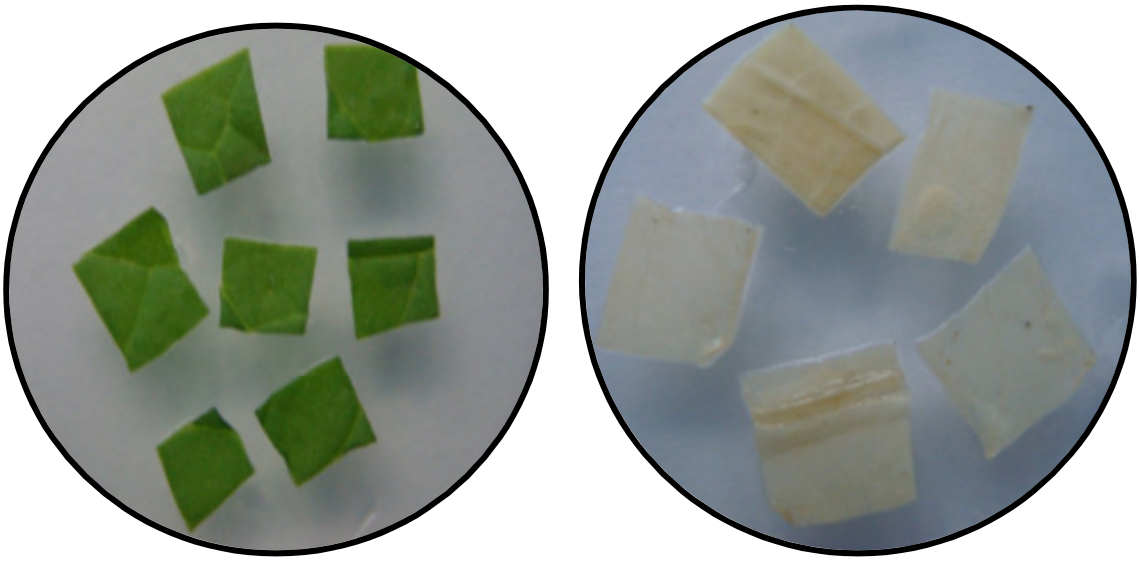


Figure 4.13: Leaf disc assay for kanamycin sensitivity.

Leaf segments from putative transgenic (A) *Brassica juncea* plantlets and from the wild type (B) plantlets suspended in modified regeneration media supplemented with 30 mg/L kanamycin.

isolated from the leaves of putative transgenic plants and from wild type plants.

Quality of the isolated genomic DNA was determined by agarose gel electrophoresis (Figure 4.14) and the concentration and purity was determined spectrophotometrically (Table 4.5).

Table 4.5: Quantification of genomic DNA isolated from wild type and transgenic *Brassica juncea*

Sample	A_{260}	A_{280}	A_{260}/A_{280}	Conc. Of DNA µg/µl
Putative transgenic *Pjapx1-1*	0.560	0.302	1.850	2.80
Putative transgenic *Pjapx1-2*	0.406	0.223	1.82	2.03
Putative transgenic *Pjapx1-5*	0.508	0.288	1.76	2.54
Putative transgenic *Pjapx1-9*	0.630	0.351	1.79	3.15
Putative transgenic *Pjapx1-11*	0.497	0.262	1.89	2.48
Wild type *PjWT-1*	0.577	0.325	1.77	2.88

4.2.4.2.2 PCR Analysis

Genomic DNA isolated from five independent transgenic plants and one wild type (control) plant was used as template DNA for PCR amplification by *nptII* and *apx1* cDNA specific primers. Colony PCR with respective primers was used as a positive control. The presence of a band of 734 bp in samples from transformed plants (lanes 3, 4, 5, 6 and 7) confirmed the integration of the *nptII* gene (Figure 4.15). No PCR product was observed in wild type (control) plants (lane 2). Also the presence of a band at 898 bp in samples from transformed plants (lanes 3, 4, 5, 6 and 7) confirmed the integration of *apx1* cDNA (Figure 4.16). Interestingly amplification of this fragment (898 bp) was not observed in wild type (control) plants (lane 2); because the *apx1* cDNA primers designed, were specific for the transgene *i.e.*, *apx1* from

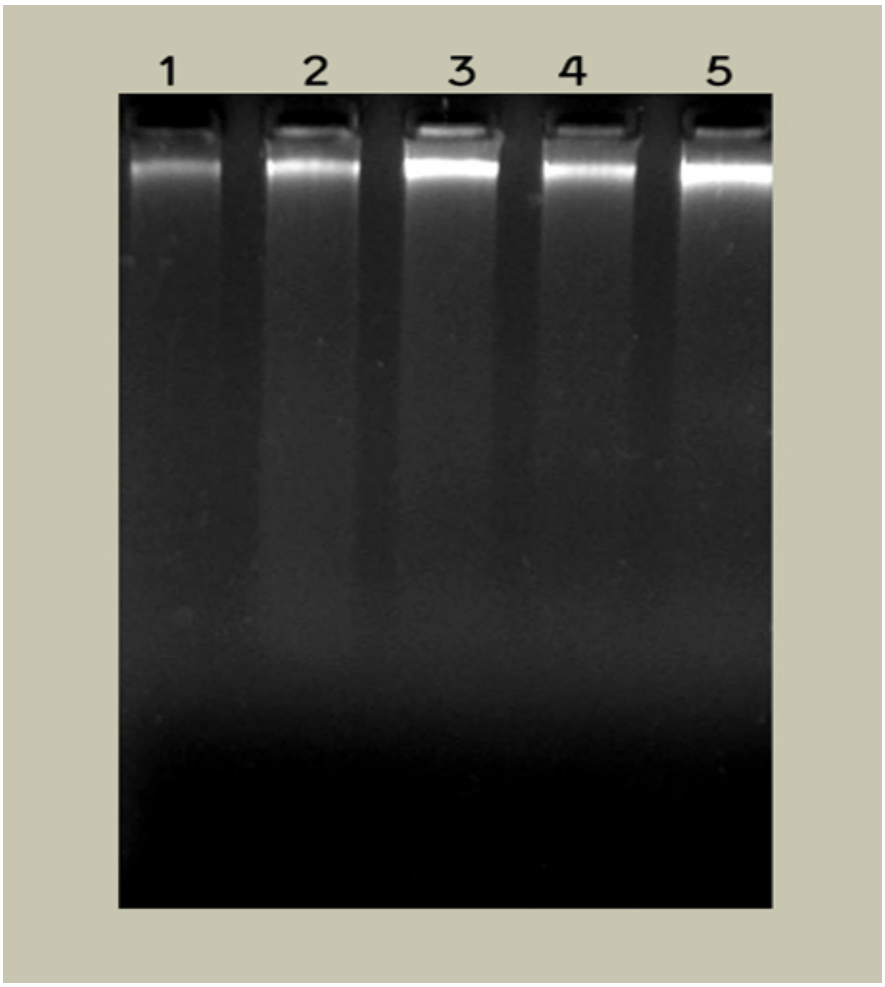


Fig. 4.14 Genomic DNA Preperation
Lane 1-5: genomic DNA from 5 independent transgenic plants.

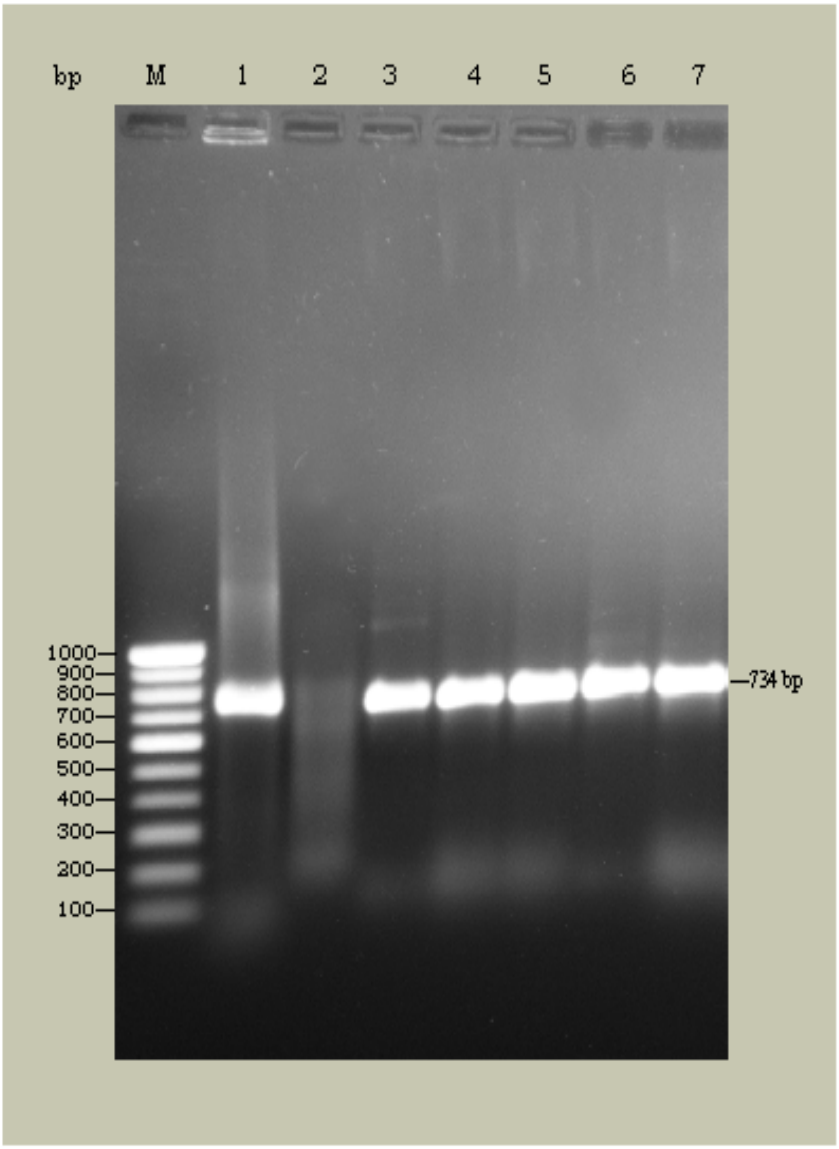


Fig.4.15 PCR analysis of putatively transformed *Brassica juncea* plantlets for the presence of *nptII* gene Lane [M]: Molecular weight marker; Lane [1]: Positive Control; Lane [2]: Negative Control(Genomic DNA from wild type plant); Lane [2-7]: Genomic DNA from 5 independent transformed plants.

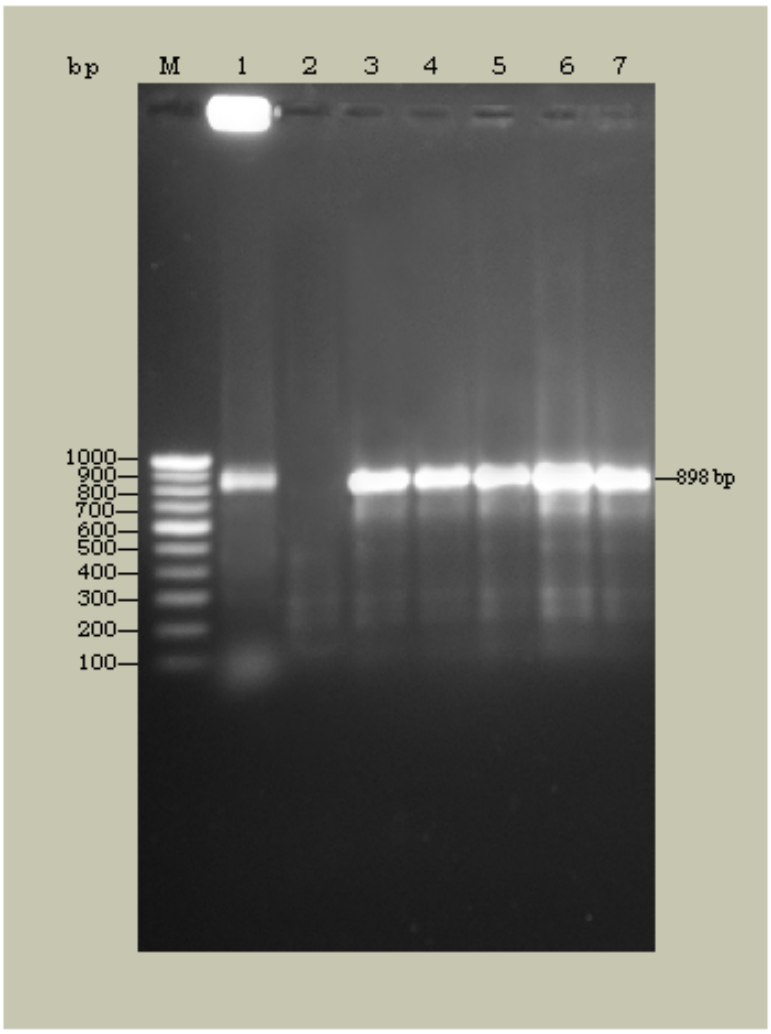


Fig. 4.16 PCR analysis of putatively transformed *Brassica juncea* plantlets for presence of *apx1* gene Lane [M]: Molecular weight marker; Lane [1]: Positive Control; Lane [2]: Negative Control (genomic DNA from wild type plant); Lane [2-7]: Genomic DNA from 5 independent transformed plant.

Arabidopsis thaliana; in other words the region from where the primers were selected had little similarity with the similar gene present in *Brassica juncea*. So from these results we can confidently conclude that the gene of interest *i.e.*, *apx1* from *Arabidopsis thaliana* has successfully been integrated into the genome of the transgenic *Brassica juncea* plantlets.

4.3 Comparative Physiological Studies of Wild Type and Transgenic Plants

Physiological status and growth of transgenic plants was compared with that of wild type plants to rule out the occurrence of any abnormality in the physiology of transgenic plant, Photosynthetic rate, stomatal conductance, chlorophyll fluorescence and Soil Plant Analysis Development (SPAD) values were measured using Infra red Gas Analyzer, plant efficiency analyzer and portable SPAD meter, respectively.

4.3.1 Photosynthetic rate and stomatal conductance

Photosynthetic efficiency in plants is a result of interaction among different factors like CO_2 concentration, ambient temperature, photon flux density, chlorophyll content, water and nutrient status etc. Stomatal conductance is well correlated with the gas exchange and CO_2 assimilation **(Salisburry and Ross, 1992; Taiz and Zeiger, 2004)** which ultimately affect photosynthetic rate and photosynthetic rate in turn is linked with the productivity of plant. There have been many reports about the positive relationship between photosynthesis and stomatal conductance **(Johnson *et al.*, 1987, Suresh *et al.*, 1997).**

During the vegetative stage, gas exchange measurements were conducted with the young fully expanded leaves of wild type as well as transgenic plants. Photosynthetic parameters, including net

photosynthetic rate (*Pn*), stomatal conductance (*Gs*) were assessed. In the present investigation no significant difference was found in net photosynthetic rate (Figure 4.17) and stomatal conductance (Figure 4.18) of Wild type and transgenic plant; revealing that transgenic plant showed normal photosynthesis viz a viz wild type plants.

4.3.2 Chlorophyll fluorescence

Chlorophyll fluorescence measurements are widely used for analyzing the health integrity of photosynthetic apparatus and the effect of external environmental factors on photosynthetic activity. Chlorophyll fluorescence is also frequently used as a potential indicator of environmental stress and a potential screening method for stress tolerant plants. The Fv/Fm ratio can be considered as a measure of the quantum efficiency of the electron transport in photosystem II (PSII) **(Maxwell and Johnson, 2000).** Where, Fm is the maximal chlorophyll fluorescence when all PSII reaction centres are closed in dark-adapted leaves and Fv is the variable fluorescence after dark acclimation. Higher the variable fluorescence, higher is the photosynthetic capacity of the leaf (**Lichtenthaler, 1990**). Thus the parameters maximal fluorescence (Fm), variable fluorescence (Fv) and the Fv/Fm ratio characterize the functional state of PSII. To reliably estimate the physiological status of plants by the fluorometric method, it is necessary to measure the Fv/Fm ratio accurately.

A slight difference of 6.49% was observed in the chlorophyll fluorescence (Fv/Fm) of the wild type and transgenic plants (Figure 4.19). The chlorophyll fluorescence Fv/Fm ratio of transgenic plants was 6.49% lower than wild type plants. This difference, through statistically insignificant, could be caused because of the expression of ascorbate peroxidase enzyme, which might be effective in the physiology of transgenic plants. The chlorophyll fluorescence

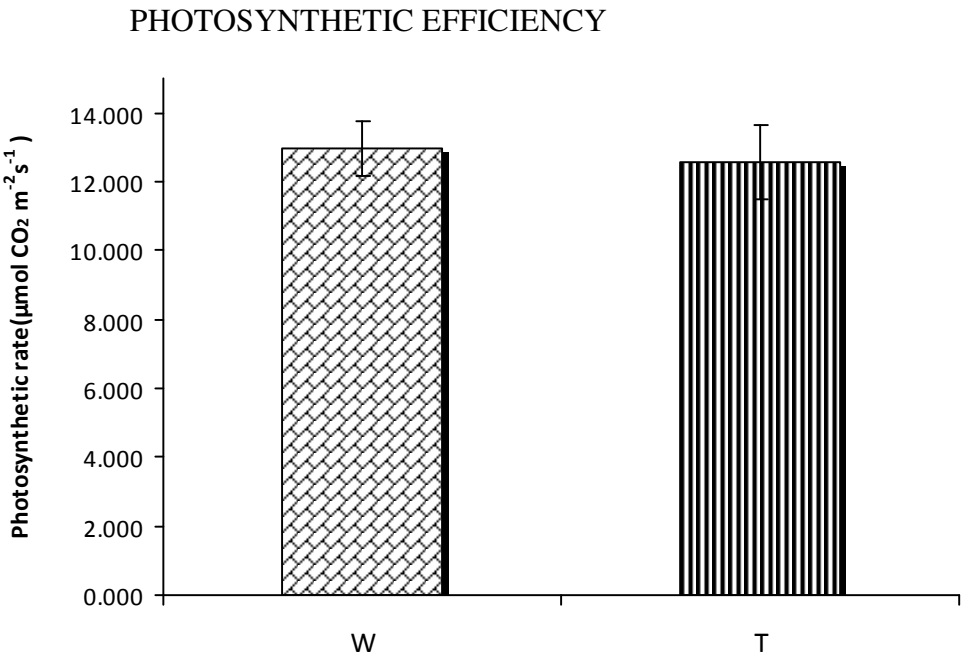


Fig. 4.17 Photosynthetic rate of wild type (W) and transgenic (T) Brassica juncea plants. Data plotted are an average of three independent experiments with three replicates each. Line above bars Mean ± SE.

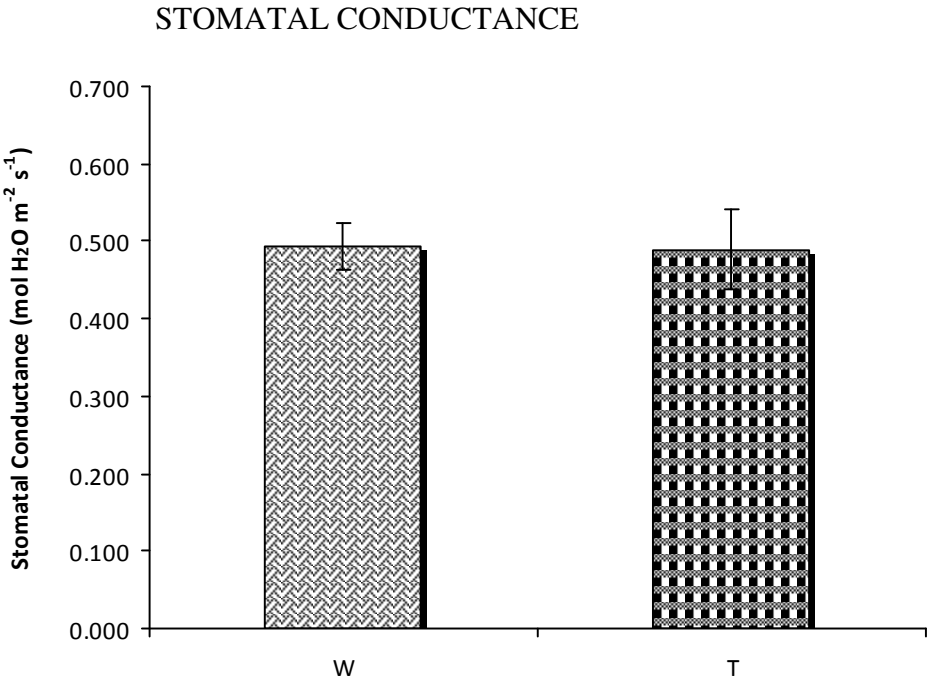


Fig. 4.18 Stomatal Conductance of wild type (W) and transgenic (T) Brassica juncea plants. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

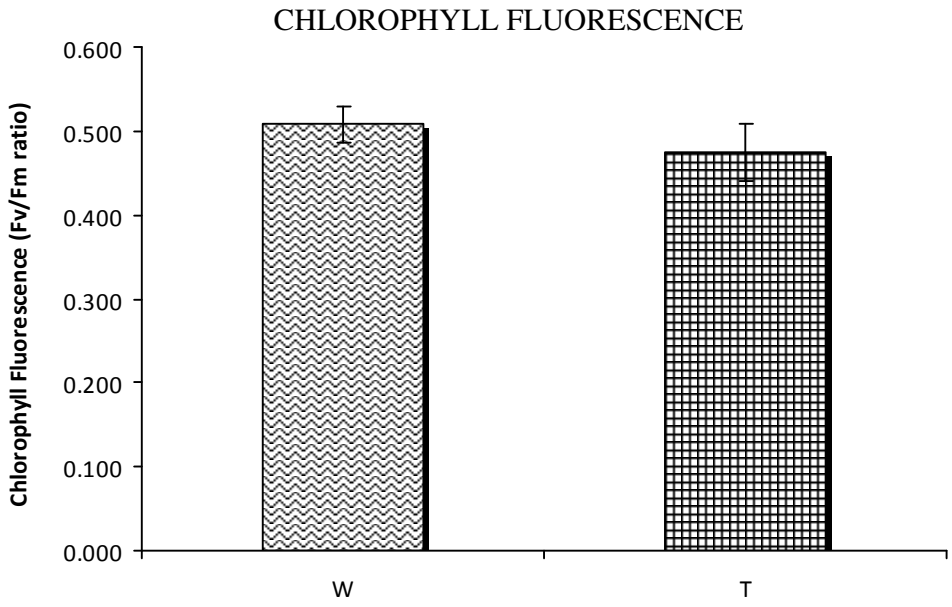


Fig .4.19 Chlorophyll fluorescence (Fv/Fm ratio) of wild type (W) and transgenic (T) Brassica juncea plants. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

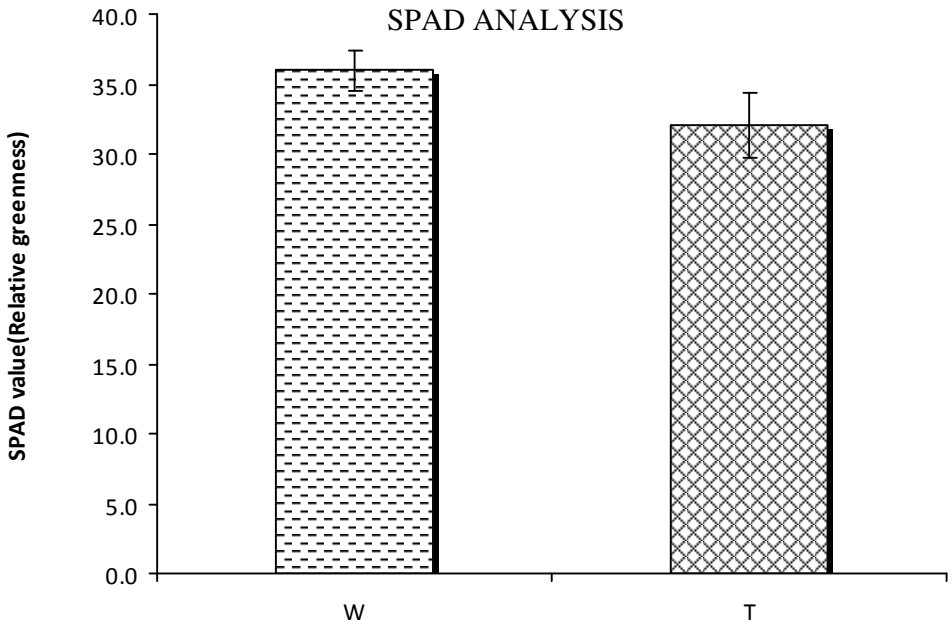


Fig. 4.20 SPAD value of wild type (W) and transgenic (T) *Brassica juncea* plants. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

induction method is widely used for the examining the physiological health of plants under various environmental stresses (**Havaux, 1993; Schreiberg *et al.*, 1995**). Our data suggests that the photosynthetic efficiency of transgenic plants was comparable to the wild type plants under control conditions and no more change in the architecture of the photosynthetic apparatus of transgenic plants are expected.

4.3.3 Soil Plant Analysis Development (SPAD) value:

The SPAD chlorophyll meter determines the relative amount of chlorophyll in leaves or greenness of plant by measuring transmittances at red (650 nm, where absorption is high) and near-infrared (940 nm, where absorption is extremely low) wavelength regions **(Minolta, 1989)**. The light transmitted by the leaf is converted into electrical signals and the ratio of the intensities of transmitted light, at the two wavelengths provides the SPAD reading. Researchers have widely used SPAD meter readings to predict chlorophyll concentration because of the high correlation with chlorophyll concentration on a per unit leaf area basis **(Peng *et al.*, 1993).**

SPAD values were measured in young fully expanded leaves of wild type and transgenic plants. Relative greenness of wild type plants was recorded to be better than the transgenic plant under normal conditions of growth and soil; (Figure 4.20). The lower values for transgenic plants (11%) could be attributed to the diversion of carbon and metabolic energy towards the continuous synthesis of ascorbate peroxidase and possible changes in the flavonoid and carotene contents of plants.

Thus from the above studies it can be concluded that the physiological and growth status of transgenic plant was comparable to that of the wild type plants, although the wild type plants hold a slight edge over the transgenic plants. So it can be safely assumed that

introgression of *apx1* construct did not significantly alter the normal physiological status of transgenic plants.

4.4 Salinity Stress Tolerance Profiling of Transgenic *Brassica juncea*

For evaluating the oxidative stress tolerance potential of transgenic plants and to compare it with that of the wild type plants, various standardized biochemical parameters were assayed under salinity induced oxidative stress.

4.4.1 Salinity stress treatment

Leaf segments from transgenic and wild type plants were subjected to salinity stress by suspending them in liquid MS media supplemented with 0 and 200 mM NaCl. Ten leaf segments per treatment, from transgenic and wild type *Brassica juncea* plants were separately floated in petri dishes containing 20 ml of medium without and with 200 mM NaCl. All treatments were incubated for 24 hours under continuous light having a photon flux density of approximately 21,500 lux at 25± 2 ^{o}C **(Mishra *et al.*, 2005)** After 24 hours of incubation the leaf segments were used as experimental material for oxidative stress analysis (Figure 4.21, 4.22).

4.4.2 Proline accumulation

Osmotic adjustment in plants, subjected to salinity stress, can occur through the intracellular accumulation of either inorganic ions or low molecular weight organic solutes. Although both of these play a crucial role in higher plants, grown under saline conditions, their relative contribution varies from species to species **(Greenway *et al.*, 1980, Ashraf, 1994)**. The compatible osmolytes generally found in higher plants are low molecular weight sugars, organic acids, polyols and nitrogen containing compounds.

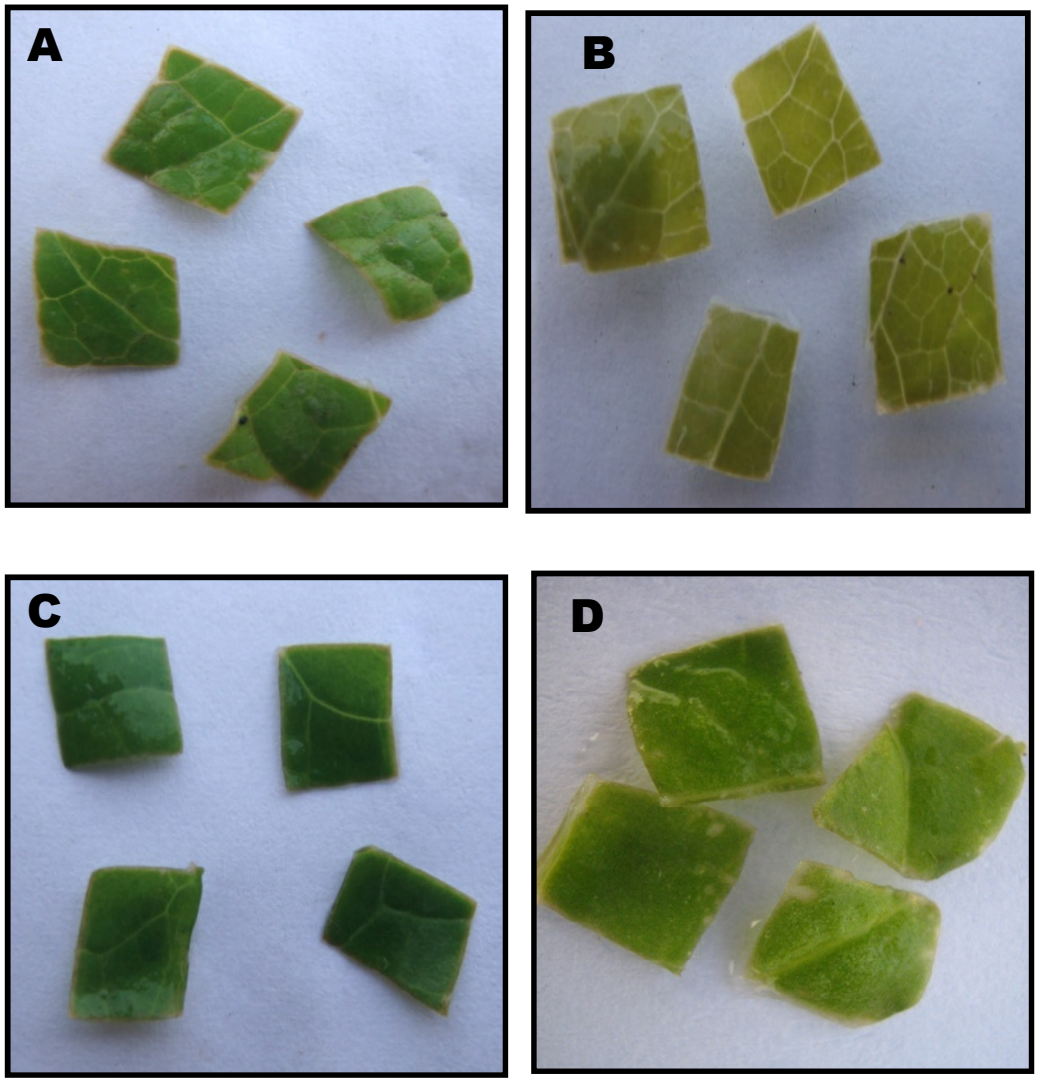


Fig. 4.21 Salinity stress Treatments

A) Leaf segments from wild type plants suspended in liquid MS medium without 200 mM NaCl.

B) Leaf segments from wild type plants suspended in liquid MS medium supplemented with 200 mM NaCl

C) Leaf segments from transgenic plants suspended in liquid MS medium without 200 mM NaCl.

D) Leaf segments from transgenic plants suspended in liquid MS medium supplemented with 200 mM NaCl

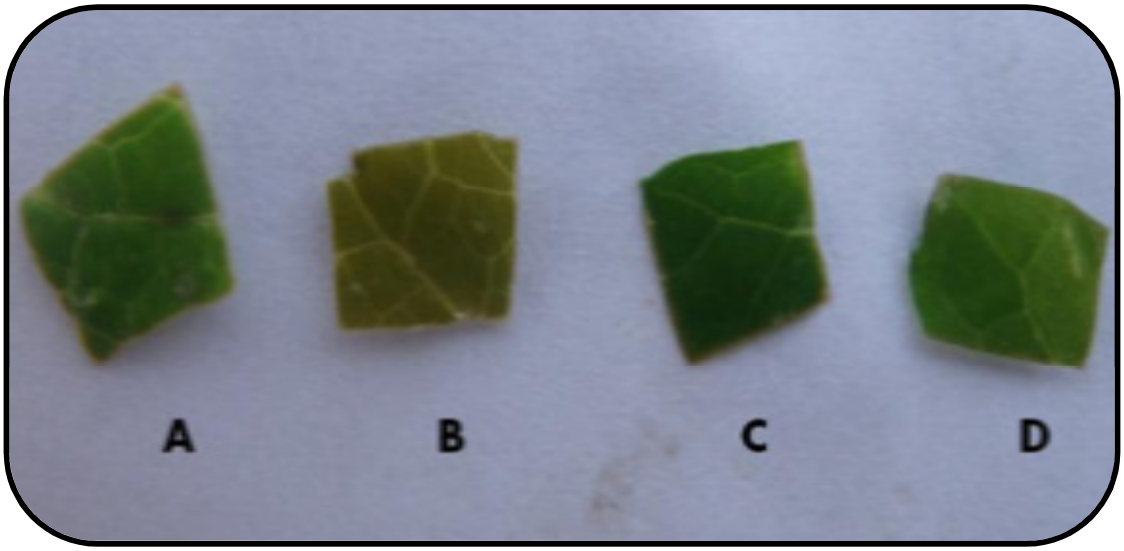


Figure 4.22 Comparison of stress treatment results in a close view

A: Leaf segment of wild type plants in liquid MS medium without 200 mM NaCl

B: Leaf segment of wild type plants in liquid MS medium with 200 mM NaCl

C: Leaf segment of transgenic plants in liquid MS medium without 200 mM NaCl

D: Leaf segment transgenic plants in liquid MS medium with 200 mM NaCl

Proline occurs widely in higher plants, and preferentially accumulates in higher concentrations than any other amino acid under salinity stress **(Ali *et al.*, 1999, Abraham *et al.*, 2003)**. Rapid accumulation of free proline is a typical response to salt stress. In organisms ranging from bacteria to higher plants there is a strong correlation between increased cellular proline accumulation and the capacity to survive, under water deficit stress **(Ahmad and Jhon, 2005)**.

Under salt stress treatment, proline content increased in both wild type and transgenic leaf segments; however net proline content of transgenic leaves was significantly higher than the wild type leaf segments, in presence of 200 mM NaCl. Although under control conditions also the proline content in the leaf segments of transgenic plants was 1.26 fold higher than the wild type leaf segments, however after 24 hrs of salinity stress treatment this difference increased to 1.8 fold i.e. under stress the transgenic leaves had 77% higher proline than the wild type leaf segments (Figure 4.23). A higher proline content in transgenic leaves under control conditions could be attributed to the changes in the normal biosynthetic reactions in the cells, which are being forced to continuously express *apx1* gene introduced under the control of a constitutive promoter. However, under salt stress treatment a significantly higher proline accumulation in transgenic leaf discs could be attributed to the protective role of proline as an osmoprotectant as well as a free radical scavenger (**Matysik *et al.*, 2002**).

Proline content have been reported to increase under NaCl stress in *Phaseolus aureus* **(Misra and Gupta, 2005)**, *Morus alba* **(Ahmad *et al.*, 2007)** and *Sesamum indicum* **(Koca *et al.*, 2007)**. Apart from protection of macromolecules from denaturation and serving as carbon and nitrogen reserve for stress relief, proline has

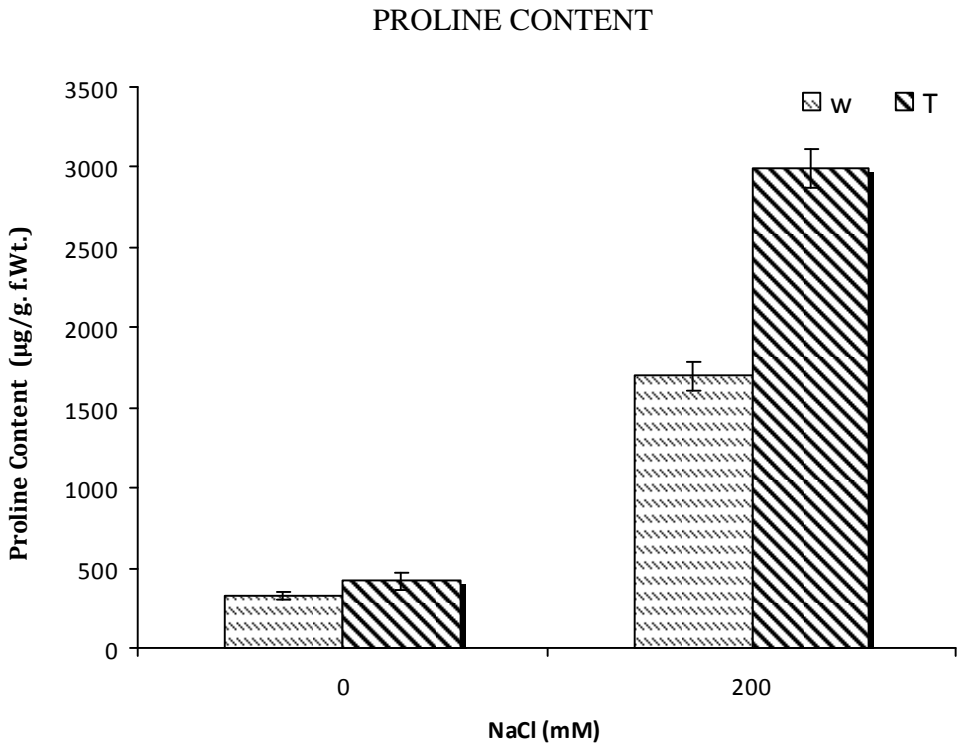


Fig. 4.23: Proline content in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

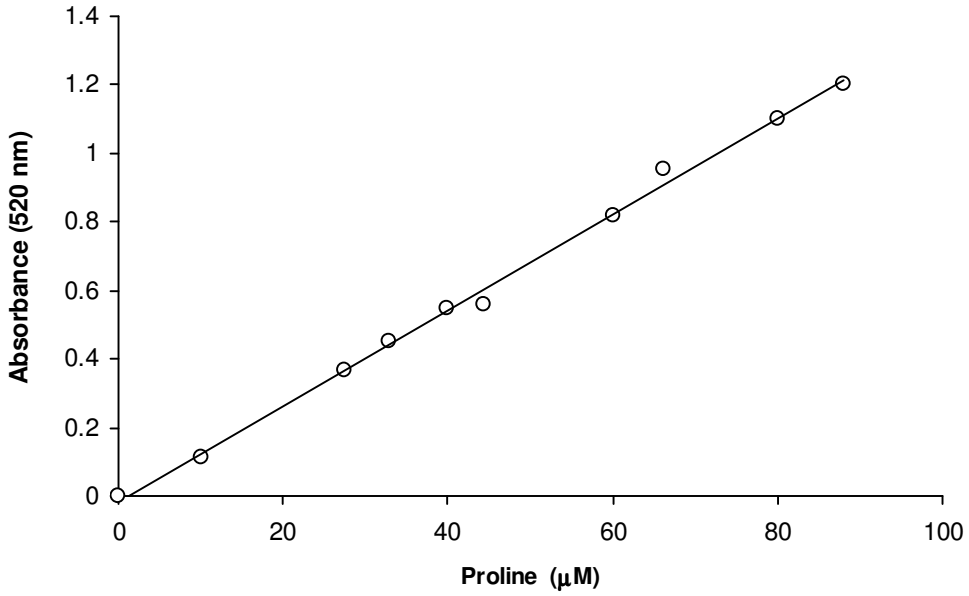


Figure 4.24: Standard curve of L-proline

several other functions during stress: e.g. osmotic adjustment **(Voetberg and Sharp, 1991)**, osmoprotection **(Kishor *et al.*, 2005)**, free radical scavenger and as an antioxidant **(Sharma and Dietz, 2006)**. A positive correlation between magnitude of free proline accumulation and salt tolerance has been suggested as an index for determining salt tolerance potentials **(Ramanjula and Sudhakar, 2001)**.

In our experiments the principal reason for the accumulation of proline in leaf segments, stressed with NaCl is the physiological drought caused by NaCl, which activates the genes of proline biosynthesis and repressed those of its degradation **(Hopkins, 1995)**. Similar results have been reported for *Cicer arietinum* **(Soussi *et al.*, 1999)** and *Oryza sativa* **(Sultana *et al.*, 1999)**.

Proline concentration in many salt tolerant plants has been found to be higher than that in salt sensitive ones. **Petrusa *et al.*, 1997** found that salt tolerant alfalfa plants rapidly doubled their proline content in the roots, whereas in salt sensitive plants the increase was slower and lesser. Similar results were also reported in alfalfa **(Fougere *et al.*, 1991).**

Even in case of *Brassica juncea*, relatively salt-tolerant plants showed a higher degree of osmotic adjustment in the leaves and a higher critical point concentration of NaCl, at which the endogenous level of free proline rose sharply, than did the relatively salt sensitive genotypes **(Jain *et al.*, 1997)**. Higher proline accumulation was found in salt-tolerant *B. juncea* plants with better growth potential than the control ones **(Kirti *et al.*, 1991)**. Furthermore, in *B. juncea* plants grown under stress conditions, activities of proline biosynthetic enzymes P5CR and ornithine-aminotransferase (OAT) increased significantly though the activity of proline degrading enzyme "proline oxidase" was lowered **(Madan *et al.*, 1995)**.

Hence increased accumulation of Proline in leaf segments from transgenic plants may be linked to increased tolerance to salinity stress.

4.4.3 Malondialdehyde content

A direct result of stress-induced cellular changes is the enhanced accumulation of toxic species in cells that includes various activated oxygen species (**Suzuki and Mittler 2006).** A direct quantification of reactive oxygen species generated under stress in vivo is difficult, and most researchers have routinely used the production of Malondialdehyde content (MDA) as an indicator of ROS induced damage **(Chen and Murata, 2002).** The peroxidation of membrane lipids in plants exposed to adverse environmental conditions is a reliable indicator of free radical generation and accumulation in the tissues. The content of lipid hydroperoxidase is estimated as MDA content, which is a product of lipid peroxidation in living tissues. Therefore malondialdehyde content is used as a biomarker to estimate the extent of damage to cell membranes **(Xiao *et al.*, 2008).**

MDA content in leaf segments of transgenic and wild type *Brassica juncea* plants subjected to salinity stress are shown in Figure 4.25. Leaf segments from both types of plants recorded an increase in lipid peroxidation level under 200mM NaCl stress. After 24 hrs of incubation the transgenic leaf segments showed only a 1.9 fold increase under stress while the wild type leaf segments showed a 2.6 fold increase over the control leaf segments, respectively. Thus it is clear that leaf segments from transgenic plants have higher protective activity than the leaf segments from wild type plants, under similar stress conditions; signifying that transgenic plants have better capacity to detoxify ROS in comparison to wild type.

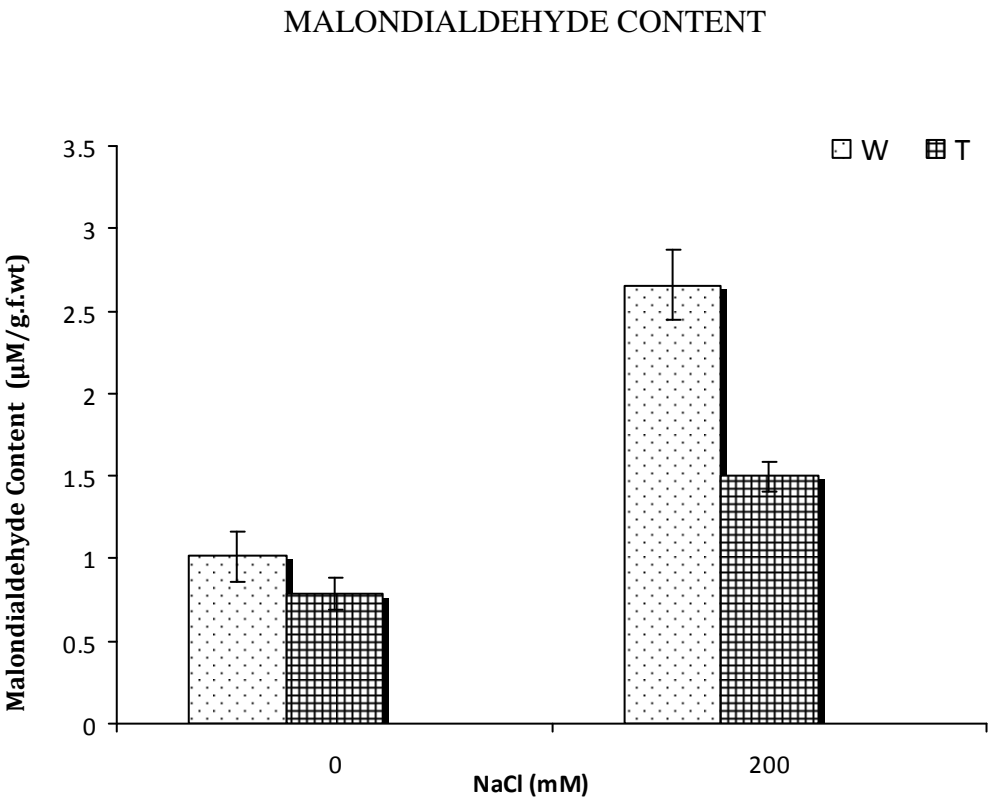


Fig. 4.25: MDA content in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with two replicates each. Line above bars represents Mean ± SE.

A lower level of lipid peroxidation and consequently a lower degree of membrane damage in genetically engineered *Brassica juncea* plants than in wild type might be due to the improved defense system of the transgenic plants. It has been documented that salinity induces oxidative stress in plant tissues and lipid peroxidation has frequently been used as an indicator of oxidative stress in plants subjected to salinity. This has been shown for *Morus alba* **(Sudhakar *et al.*, 2001),** *Lycopersicon esculentum* **(Mittova *et al.*, 2002),** *Beta vulgaris* **(Bor *et al.*, 2003)**, *Oryza sativa* **(Vaidyanathan *et al.*, 2003)** and *Gossypium hirsutum* **(Meloni *et al.*, 2003).**

Vendruscolo *et al.* (2007) reported that MDA content in non transgenic plants was 65% higher when compared to transgenic plants of wheat during water stress. **Koca *et al.* (2007)** also showed that lipid peroxidation was higher at 100 mM NaCl treatment in salt sensitive cultivar of *Sesamum indicum* than salt tolerant one. Over-expressing the *B. juncea* dehydrins enhanced stress tolerance by suppressing the MDA content in transgenic tobacco (**Xu *et al.*, 2008**).

Thus our results indicate that transgenic plants have higher capability of plant protection against oxidative damage caused by salt treatment. This protection might result from significantly higher constitutive over-expression of cytosolic ascorbate peroxidase in the transgenic plant.

According to **Scandalios (1993)** catalase and ascorbate peroxidase are the antioxidant enzymes most effective in preventing cellular damage. A lower lipid peroxidation resulting from elevated activities of antioxidant molecules under salt stress has also been reported in salt-tolerant wild tomato species **(Shalata *et al.*, 1998)**, pea cultivars **(Bor *et al.*, 2003)**, eggplant **(Yasar, 2003)**, and in sugar beet **(Hernandez *et al.*, 2000)**.

4.4.4 Hydrogen peroxide content

In the present experiments leaf segments of transgenic plants, showed 3.9 fold increase in hydrogen peroxide contents under 200 mM NaCl stress, in contrast to the wild type leaf segments that showed a 6.1 fold increase over the respective controls (Figure 4.26). Interestingly, even under control condition (0 mM NaCl), the leaf segments of transgenic plants showed 25% less H_2O_2 than the leaf segments of wild type plants however, under 200 mM NaCl leaf segments of wild type plants accumulated 95% higher H_2O_2 than the leaf segments of transgenic plants. The lower accumulation of H_2O_2 content in leaf segments of transgenic plants, with or without salinity treatment, can be directly correlated with increased activity of cytosolic ascorbate peroxidase as a result of over-expression of *apx1* c DNA construct.

It is well known that salt stress can cause excessive accumulation of ROS, including hydrogen peroxide (H_2O_2), resulting in oxidative damage to cells **(Sairam and Tyagi, 2004)**. The H_2O_2 formed as a product of superoxide dismutase activity is a potential damaging agent, especially at higher salt concentrations. It has been observed that H_2O_2 can easily penetrate complex multilayered biological membranes, since the pka of H_2O_2 is 11.8 and H_2O_2 exists in a neutral form at physiological pH **(Takeda *et al.*, 1995)**. H_2O_2 can also react with superoxide radical to form highly reactive hydroxyl radicals. These hydroxyl radicals initiate self- propagating reactions leading to peroxidation of membrane lipid and destruction of proteins and may finally lead to cell death **(Jaw and Ching, 1998; Sairam *et al.*, 2000).**

Due to the extreme reactivity and excessive mobility of H_2O_2, it is very much essential to metabolize H_2O_2 immediately so as to prevent its accumulation. H_2O_2 formed in the chloroplasts and mitochondria

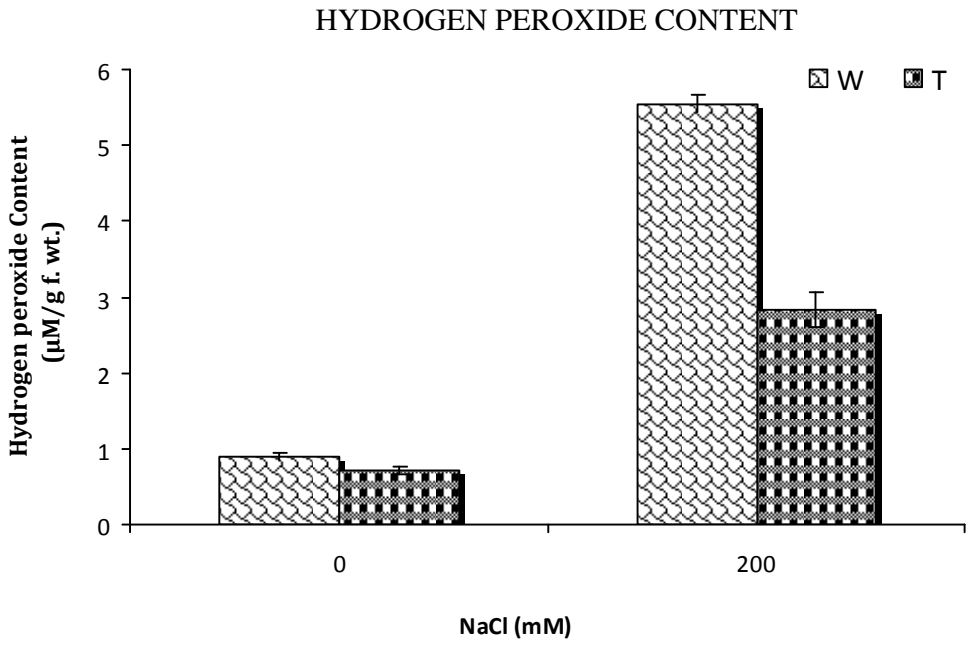


Figure 4.26: Hydrogen peroxide content in the leaf discs of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with two replicates each. Line above bars represents Mean ± SE.

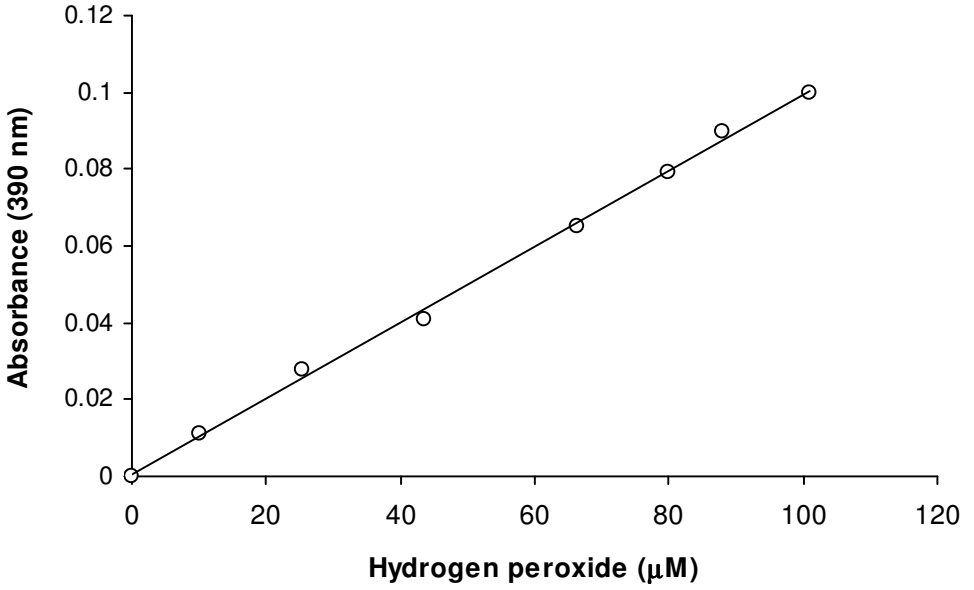


Figure 4.27: Standard curve of Hydrogen peroxide

diffuses from these organelles into the cytosol and is then scavenged by AsA-GSH cycle, which includes ascorbate peroxidase located in the cytosol **(Ishikawa *et al.*, 1993; Takeda *et al.*, 1995)**.

Lu *et al.* (2007) have also observed a higher accumulation of H_2O_2 in wild type *Arabidopsis* than the transgenic ones over-expressing rice cAPX, in response to salinity stress. **Sairam and Srivastava, 2000** found that tolerant genotypes possessing higher APX activity had lower H_2O_2 content than the susceptible genotypes, under high temperature stress. Therefore the results obtained in the present experiment clearly validate the protective role of ascorbate peroxidase under salinity (oxidative) stress and support the hypothesis that strengthening of the antioxidative defense pathway, can be used to develop broad spectrum stress tolerant genotypes.

4.4.5 Chlorophyll & Carotenoid Content

In the present investigations chlorophyll a, chlorophyll b, Total chlorophyll, chlorophyll a/b ratio, carotenoid content and chlorophyll stability index were measured in leaf segments of wild type and transgenic plants subjected to 200 mM NaCl stress.

The chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations decreased significantly in the leaf subjected to 200 mM NaCl in both wild type and transgenic plants; however such a decrease was more profound in wild type leaf segments than the transgenic leaf segments (Figure 4.28), a decrease in chlorophyll content under stress is caused due to a faster degradation rate of chlorophyll pigments under salt stress **(Asraf, 2003)**. **Yang *et al.* (2009)** have also reported a decrease in chlorophyll *a* and chlorophyll *b* content in *Populus cathayana* subjected to salt stress.

The total chlorophyll content decreased by 170 per cent in wild type leaf segments under 200 mM NaCl stress as compared to the

CHLOROPHYLL MEASUREMENT

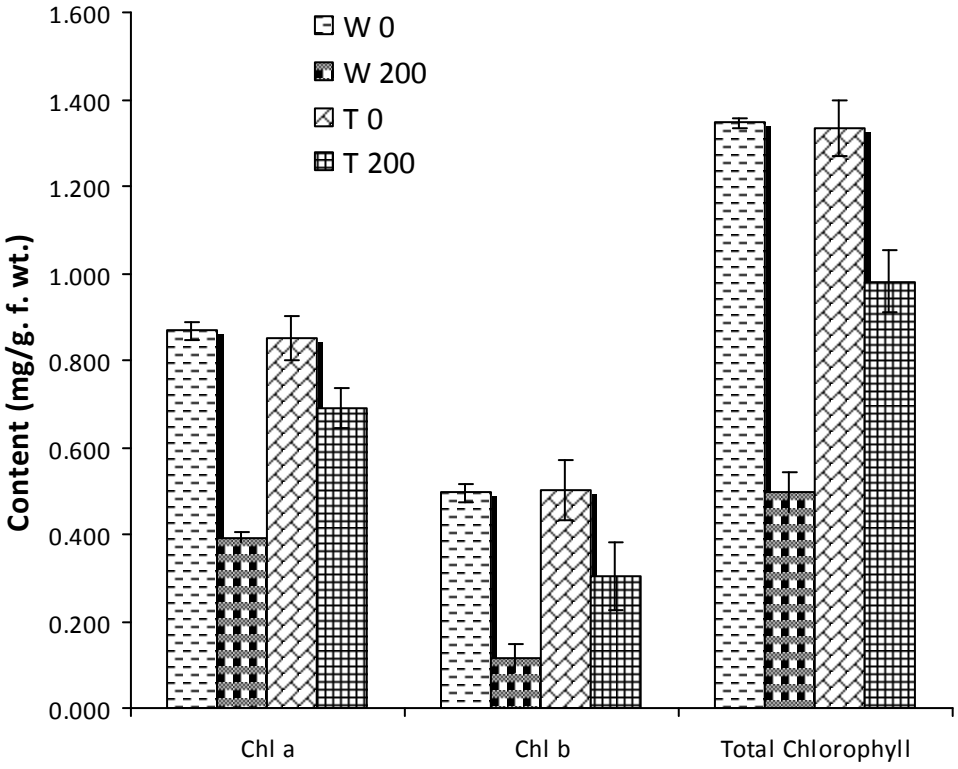


Fig. 4.28: Chlorophyll content in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with two replicates each. Line above bars represents Mean ± SE.

transgenic leaf segments which recorded only a 36 per cent decrease under 200 mM NaCl stress, over that of their respective control. (Figure 4.28). Chlorophyll concentration can be used as a sensitive indicator of the cellular metabolic status; thus, its decrease signifies oxidative stress in tissues due to accumulation of ions and other active oxygen species. Reductions of chlorophyll content under elevated salinity conditions were observed for some salt-sensitive plant species **(Ashraf *et al.*, 2000; Jungklang *et al.*, 2003; Lee *et al.*, 2004)**. While, in salt tolerant genotypes a little decrease was found **(Qui *et al.*, 2003).**

Chlorophyll a/b ratio increased in wild type as well as transgenic leaf segments under 200mM NaCl stress. Interestingly 97 percent increase in Chlorophyll a/b was observed in wild type leaf segments while in transgenic leaf segments only 34 percent increase was recorded over their respective control (Figure 4.29). In the present investigations it was observed that both chlorophyll pigment content (a&b) decreased under salt stress; with a maximum downward fluctuation recorded in chlorophyll b. It indicates that chlorophyll b is more sensitive than chlorophyll a under stress conditions **(Netondo *et al.*, 2004)**. This ultimately resulted in a higher chlorophyll a/b ratio under stress. The increase in Chl a/b ratio has been linked with the changes in pigment composition of photosynthetic apparatus which possesses lower level of light harvesting chlorophyll proteins (LHCPs) **(Loggini *et al.*, 1999)**. The reduction in LHCPs content is an adaptive defense mechanism of chloroplasts in the leaves, which allows them to endure the adverse conditions **(Asada *et al.*, 1998)**.

This indicates that wild type leaf segments were experiencing more stress than transgenic one.

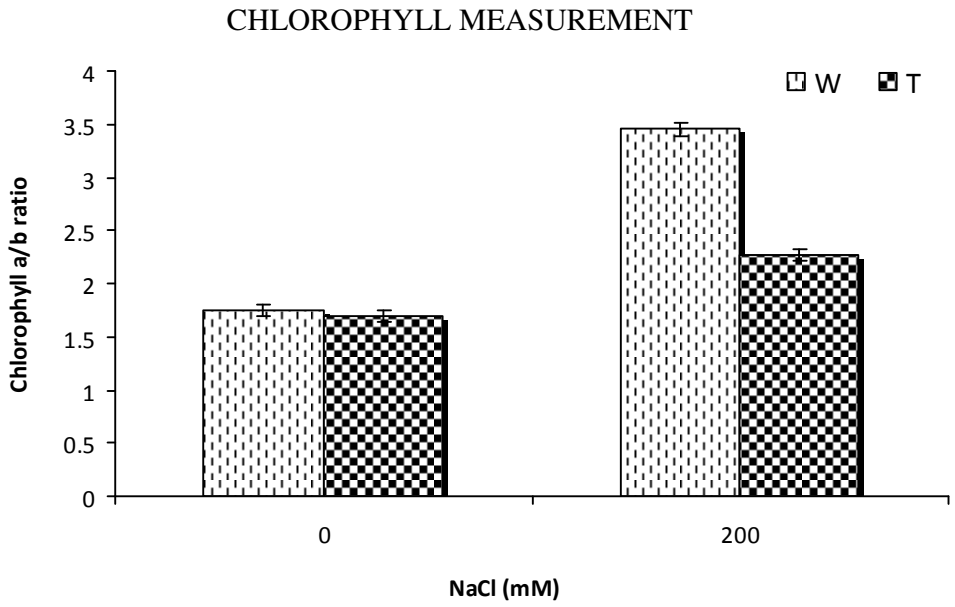


Fig. 4.29:Chlorophyll a/b ratio in the leaf discs of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

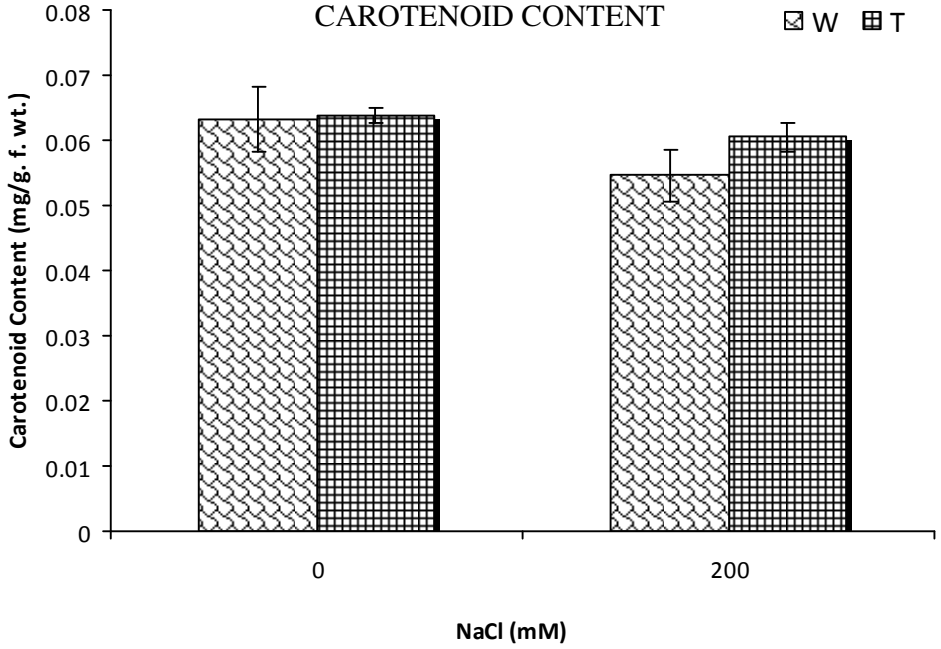


Fig. 4.30: Carotenoid content in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

Total carotenoid content decreased in both wild type and transgenic leaf segments under salinity stress; however the difference was not found significant (Figure 4.30).

Mobin *et al.*, 2007 noticed higher Chlorophyll a/b ratio under increasing Cd stress in two variety of *Brassica juncea*. The variety showing higher photosynthetic activity recorded lower Chl a/b ratio and higher APX activity than the variety showing lower phosynthetic activity but higher chl a/b ratio. In our investigations the lower Chlorophyll a/b ratio, shown by transgenic leaf segments as compared to wild type leaf segments under stress, indicate better adaptability of the transgenic leaf segments. These observations tally with the visual observations as shown in figure 4.21, 4.22.

These results are also supported by the chlorophyll stability as measured in wild type and transgenic leaf segments under 200 mM NaCl stress (Figure 4.31). Chlorophyll stability was 1.9 fold higher in the transgenic leaf segments than the wild type. The chlorophyll stability index (CSI) is a measure of stress tolerance capacity of plants. A high CSI value means that the applied stress had only a limited effect on the chlorophyll content of plants and on the overall metabolic status of the cell. This leads to increased photosynthetic rate, more dry matter production and higher productivity **(Mohan *et al.*, 2000). Wanichananan *et al.* (2003)** also correlated high CSI to high proline content in salt tolerant rice variety. In our experiment, transgenic leaf segments accumulated higher proline content and showed higher CSI than wild type leaf segments. This indicates that the transgenic plant is photosynthetically more stable than the wild type, under salt stress. This higher stability in transgenic *Brassica juncea* under stress may be attributed to an improved antioxidant status of the transgenic plants under stress, caused by the over-expression of cytosolic APX.

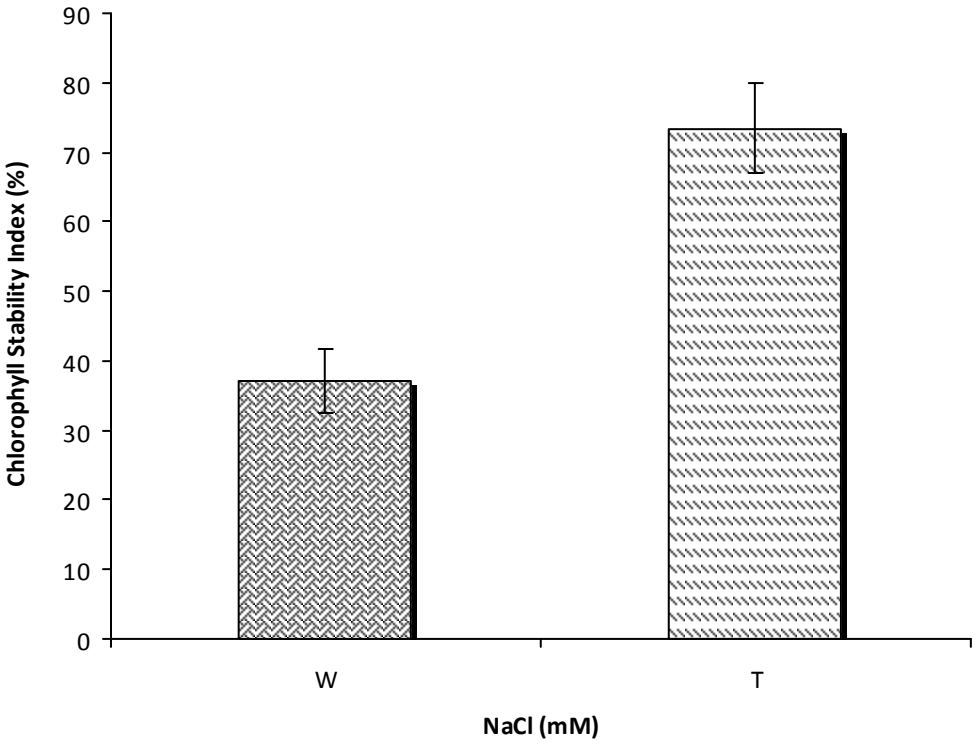


Fig. 4.31: Chlorophyll stability index (CSI) of the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

Davletova *et al.* (2005) have reported that the lack of cytosolic APX1 resulted in the oxidation of chloroplastic proteins, suggesting that APX1 activity might be important for chloroplast protection. Previous studies have shown that chloroplasts are extremely sensitive to external application of H_2O_2 **(Asada, 2000)** and the chloroplastic ascorbate peroxidase are inactivated by H_2O_2 **(Mano *et al.*, 2001)**. The lack of Cytosolic APX1 may therefore affect H_2O_2 scavenging systems in the chloroplast as well as in the whole cell; thereby underlining the crucial importance of cAPX under stress.

4.4.6 Anti-oxidative enzyme analysis

Development of oxidative stress is an inevitable consequence in plants exposed to a variety of environmental stresses including salinity stress. Antioxidant enzymes are the key elements in the defense mechanism of a plant. Several changes have been recorded in the activities of antioxidant enzymes in plants under salt stress. Therefore in the present investigations, experiments were conducted to study the effect of over-expression of ascorbate peroxidase on the activity of the H_2O_2 metabolizing enzymes.

4.4.6.1 Ascorbate peroxidase activity

Ascorbate peroxidase is reported to play an essential role in scavenging ROS and protecting cells against their toxic effects, in higher plants, algae, and several other organisms **(Sano *et al.*, 2001; Ishikawa *et al.*, 2003; Panchuk *et al.*, 2005; Teixeira *et al.*, 2006)**. Increased activity of different APX isoforms in response to environmental stresses, such as salinity and drought, has been reported in different plant species, indicating a possible functional specialization of the respective isoenzymes in eliminating H_2O_2 from cells **(Sharma and Dubey 2005; Tsai *et al.*, 2005; Teixeira *et al.*,**

2006). Computer simulation studies have shown that intracellular H_2O_2 concentration is mainly governed by APX **(Polle, 2001)**.

The activity of ascorbate peroxidase enzyme was analyzed in the leaf segments of wild type and transgenic plants, over expressing cytosolic APX (*apx1*) gene. The results showed that under unstressed conditions the specific activity of APX in leaf segments for transgenic plants was 1.58 fold higher than the leaf segments of wild type plants. Similarly, the specific activity of APX was 2.12 fold higher in transgenic leaf segments than the wild type leaf segments under 200 mM NaCl treatment (Figure 4.33). Further, the leaf segments of transgenic plants, showed a 94.5% increase in APX activity over that of control leaf segments, while on the other hand the wild type leaf segments under stress treatment showed a 44.9% increase in APX activity over their respective controls. It is clear that APX activity is selectively induced under salinity stress, although a distinctly higher APX activity in transgenic leaf segments is because of over-expression of *apx1* cDNA.

Thus collectively, our data support the hypothesis that increased tolerance to oxidative stress can be achieved through strengthening the enzymatic antioxidative defense, which in the present case is being mediated by over expression of *apx1*, under salinity stress.

APX plays an important role in salt stress tolerance **(Tsugane *et al.*, 1999; Azevedo Neto *et al.*, 2006)**. It has been reported that cytosolic APX transcripts as well as cytosolic APX activities are increased after salt stress treatment, in *Mesembryanthemum crystallinum*. The induction of APX clearly shows that this enzyme plays a critical role in controlling the elevated levels of increase of H_2O_2 concentration in plant cells during the initial salt-induced oxidative stress **(Slesak *et al.*, 2002)**.

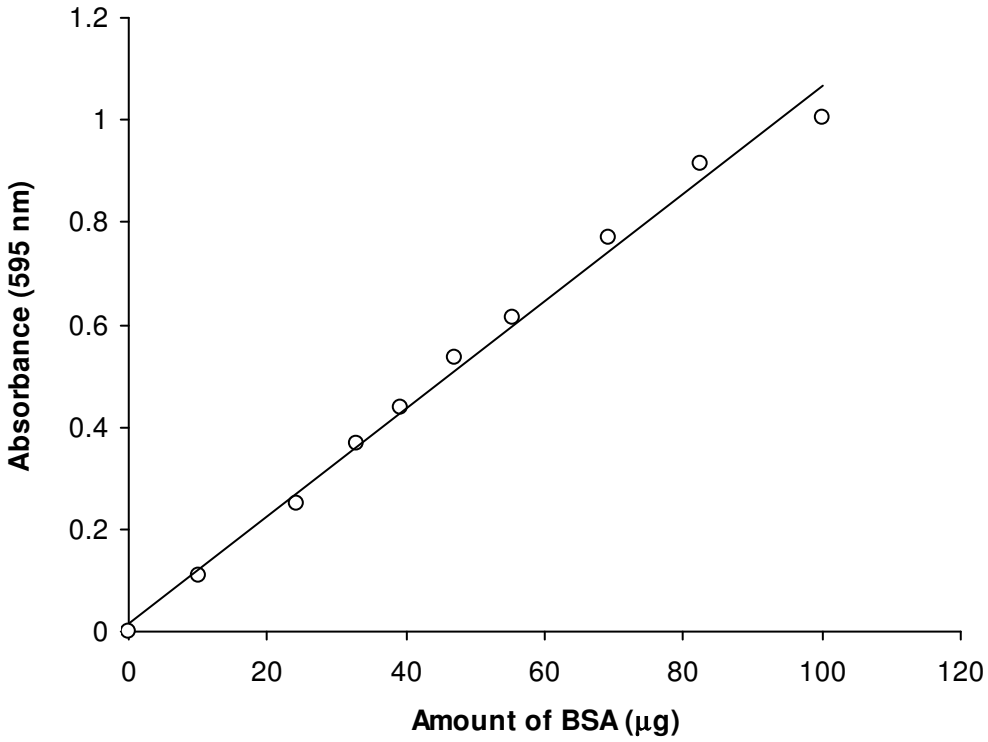


Figure 4.32: Standard curve of protein

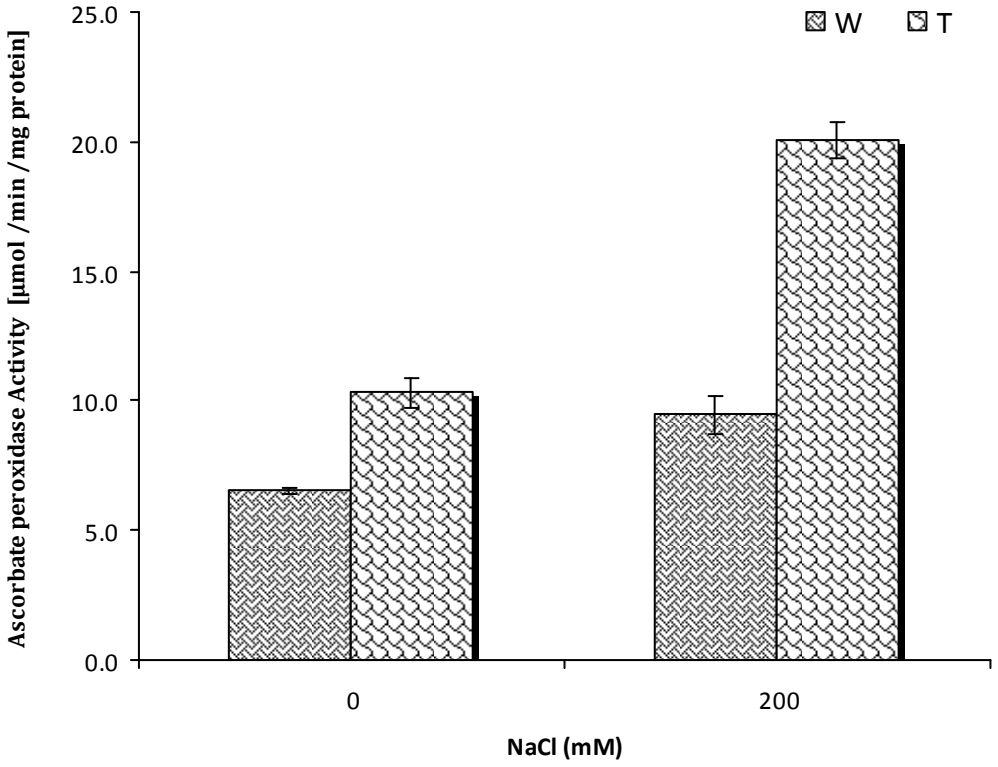


Fig. 4.33: Ascorbate peroxidase (APX) activity in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent with three replicates each experiments. Line above bars represents Mean ± SE.

A higher degree of protection against oxidative damage should require a fast and efficient removal of H_2O_2 by other scavenging systems, thus minimizing H_2O_2 toxicity and the formation of the highly toxic hydroxyl radicals **(Perl *et al.*, 1993)**. The elevated intercellular level of H_2O_2, produced under stress conditions, is reportedly regulated by catalases and peroxidases. Ascorbate peroxidase can scavenge H_2O_2 that is inaccessible for catalase, because of their high affinity for H_2O_2 and their presence in different subcellular locations **(Noctor *et al.*, 2002)**.

A number of reports suggest that over-expression of antioxidant enzymes have a cumulative effect in ROS detoxification. **Wang *et al.*, 2005** reported that increased levels of APX activity brought about by over expression of cytosolic APX gene may play an important role in ameliorating oxidative injury induced by chilling. **Lu *et al.*, 2007** noticed that transgenic *Arabidopsis* over-expressing rice Cytosolic Ascorbate peroxidase exhibited increased tolerance to salt stress compared to wild type and under normal conditions they have found nearly a two-fold increase in enzymatic activity in transgenic plants compared to wild type. The above observations tally with the results obtained in our experiments.

4.4.6.2 Guaiacol peroxidase, Total peroxidase and Catalase

The increased activities of antioxidant enzymes, on exposed to salt stress, are often directly related to the enhanced tolerance to stress. Earlier research findings have indicated that enzymes of antioxidative pathways are co regulated and over expression of one enzyme can also influence the expression activity of other enzymes **(Gueta-Dahan *et al.*, 1997; Mittova *et al*, 2004)**.

In the present study, activities of several antioxidant enzymes (CAT, GPX and POD) involved in scavenging H_2O_2, were compared in the wild type and the transgenic *Brassica juncea* leaf segments.

Under salinity stress treatment Guaiacol peroxidase activity increased in both wild type and transgenic leaf segments, however net GPX activity of transgenic leaf segments was always higher than the wild type ones, in presence as well as in absence of 200 mM NaCl. However, the leaf segments from wild type plants, subjected to 200 mM NaCl treatment, showed a 2.2 fold increase in GPX activity over the controls while the leaf discs from transgenic plants showed a 2.7 fold increase over its respective control (Figure 4.34).

Panda and Upadhayay (2003) reported an increased activity of Guaiacol peroxidase in salt stressed roots of *Lemna minor.* **Khosravinejad *et al.* (2008)** observed a linear increase in GPX activity, along with ascorbate peroxidase and catalase activity under salinity stress in barley shoots and roots.

Increased activity of GPX indicates that plants have responded positively to increased accumulation of H_2O_2, by increasing the activities of H_2O_2 scavenging enzymes; this can be attributed to increased tolerance to salinity stress.

The activity of total peroxidase showed a marked increase in the leaf segments of wild type as well as transgenic plants subjected to salinity stress. The activity of total peroxidase in the leaf segments of transgenic plants, under stress, was 2.0 fold higher than in the leaf discs of wild type plants, under similar conditions (Figure 4.35). Interestingly, the leaf discs from genetically engineered plants under stress showed 54.7 per cent higher total peroxidase activity than the control leaf discs; similarly the leaf discs from wild type plants showed 28.9 per cent increase in total peroxidase activity under stress, over the control ones. A consequently increased activity of total peroxidase

GUAIACOL PERODIXASE ACTIVITY

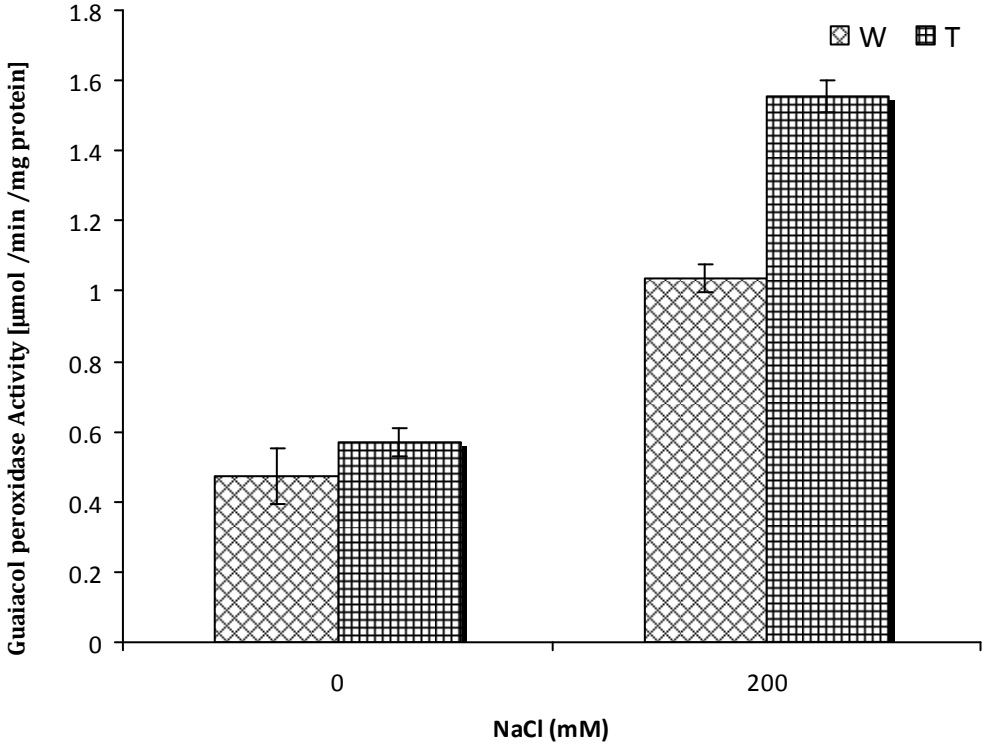


Fig. 4.34 Guaiacol peroxidase (GPX) activity in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean + SE.

TOTAL PEROXIDASE ACTIVITY

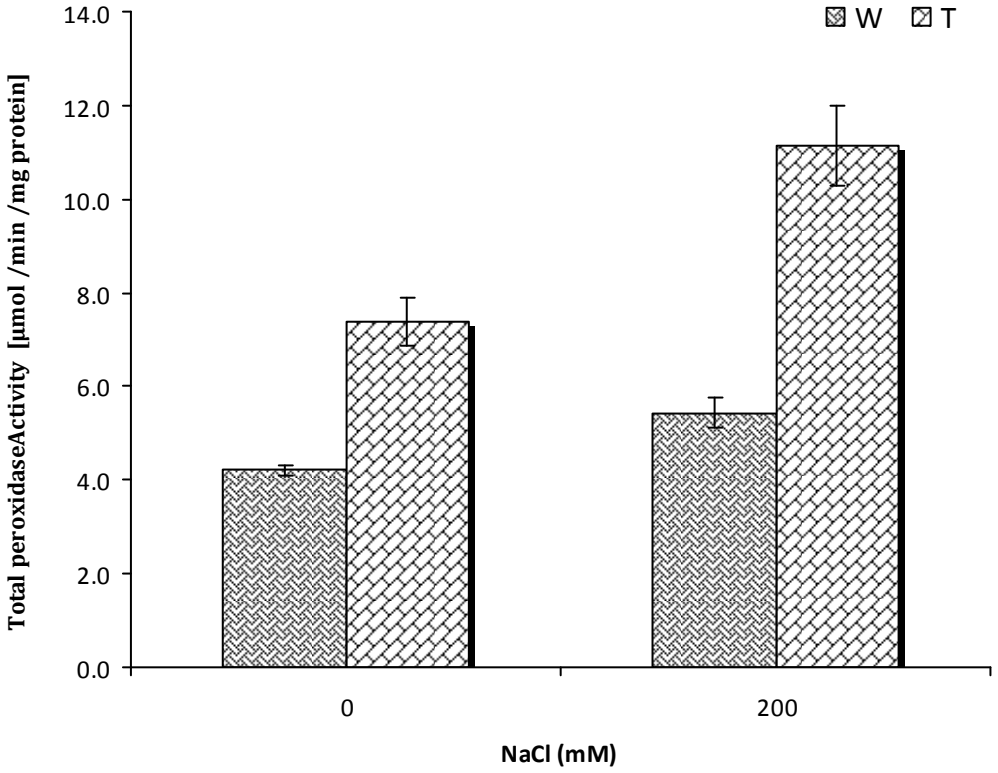


Fig. 4.35: Total peroxidase (POD) activity in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

and guaiacol peroxidase in transgenic leaf segments, under 200 mM NaCl treatment, indicate the existence of an intricate cross-talk mechanism among different peroxidases in cell.

Peroxidases are widely distributed in higher plants where they are involved in various processes, including lignification, auxin metabolism, salt tolerance and heavy metal stress **(Passardi *et al.*, 2005)**. Therefore, POD has often served as a parameter of assessing metabolism activity during growth alterations and environmental stress conditions. **Gao *et al.* (2008)** recorded increased activity of POD in *Jatropha curcas* seedlings under salinity stress**.**

Interestingly catalase activity showed a no significant difference, under control and salt stressed conditions, in wild type and transgenic leaf segments (Figure 4.36). Under stressed condition the activity of CAT in leaf segments of wild type plants recorded 11% decrease over the control segments, while the leaf segments from transgenic plant recorded a 12% decrease in its activity over the respective control.

In transgenic leaf segments the catalase activity showed no correlation with the activities of APX, GPX and POD. When exposed to salt stress, many antioxidant enzymes are reported to show alteration in their specific activity **(Lee *et al.*, 2001; Orendi *et al.*, 2001; Tsai *et al.*, 2005)**. A reported deactivation of catalase activity during salt stress may be due to the prevention of new enzyme synthesis or inactivation of catalase under stress **(Feierabend and Dehne, 1996; Polle, 1997)**. A similar decline in CAT activity has been reported in rice subjected to salt stress **(Lee *et al.*, 2001)**. However, **Yang *et al.* (2009)** reported an increased activity of catalase in *Populus cathayana* under salt stress. Interestingly no significant difference in catalase activity under control and stressed conditions was observed. **Shi *et al.* (2001) and Xu *et al.* (2008)** reported a decreased CAT activity in

CATALASE ACTIVITY

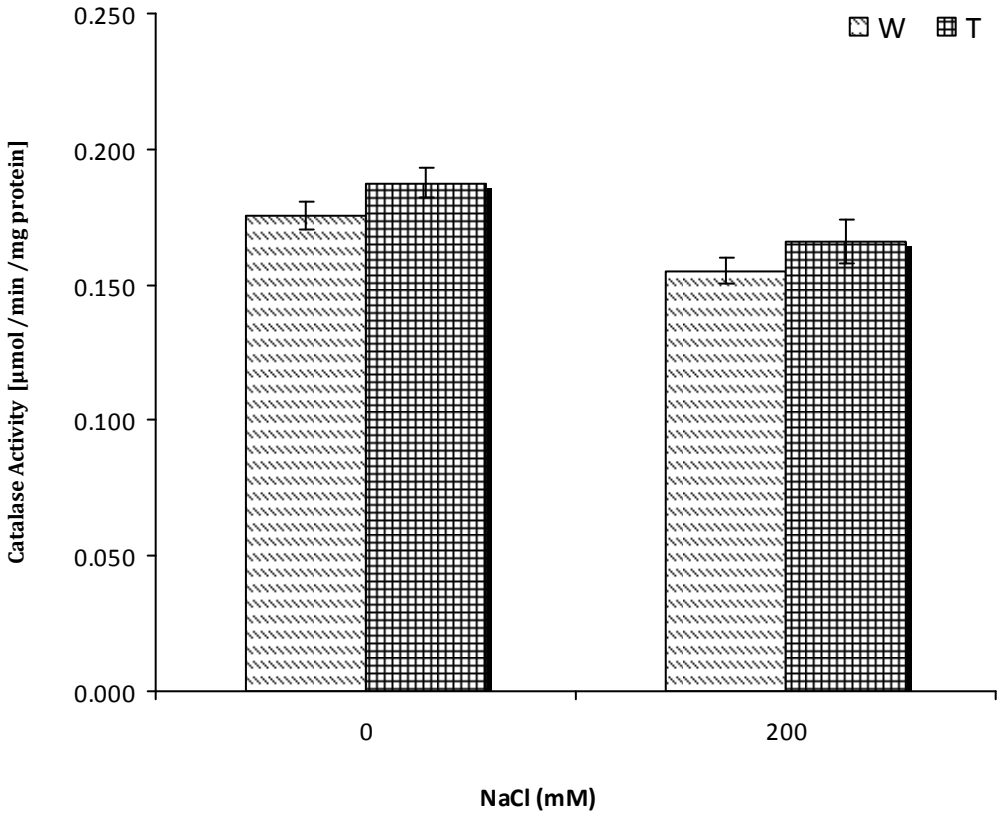


Fig. 4.36: Catalase (CAT) activity in the leaf segments of wild type (W) and transgenic (T) Brassica juncea plants, after 24 hours of incubation in liquid MS media supplemented without and with 200 mM NaCl. Data plotted are an average of three independent experiments with three replicates each. Line above bars represents Mean ± SE.

Arabidopsis plants over-expressing peroxisomal APX under heat stress and salt stress respectively.

Enzymatic scavenging of Reactive oxygen species could be efficiently achieved through the complex but elaborated coordination of various antioxidant enzymes **(Foyer and Noctor, 2000; Apel and Hirt, 2004).** Salinity stress leads to the production and accumulation of excessive amounts of H_2O_2, APX alone is not responsible for its detoxification, and other antioxidative enzymes like GPX, Catalase and POD are equally important. In the present investigations apart from increased activity of APX, increased activities of GPX and POD are also responsible for tolerance of transgenic *Brassica juncea* to salinity stress.

It can be concluded that, under normal conditions, salt stress seriously damages the growth of wild-type *Brassica juncea*; however, the expression of *apx1* enhances the limits of salt tolerance in transgenic plant(s) as evident from the biochemical and enzymatic parameters. Chlorophyll parameters further support the improved productivity of transgenic plants under salinity stress. On the basis of overall results obtained in our experiments one can safely conclude that over-expression of *apx1* in *Brassica juncea* plays a pivotal role in preventing the excess accumulation of reactive oxygen species and helps in enhancing the stress tolerance potential of host plant. However, a clear cut co-relation between various antioxidant enzymes in a cell needs further investigations.

Summary and Conclusion

Chapter 5 SUMMARY AND CONCLUSION

Agricultural productivity is severely affected by soil salinity. Salinity is one of the most serious factors limiting the productivity of agricultural crops, most of which are sensitive to the presence of high concentrations of salts in the soil. About 20% of irrigated agricultural land is adversely affected by salinity.

Hence, identification and validation of genes for imparting stress tolerance and development of salt-tolerant genotypes has become a major target of plant breeders. Although the success obtained through conventional plant breeding methods have been encouraging, but the progress has been slow and not very precise. With the advent of genetic engineering, development of new and improved genotype with specific genetic constitution for a particular agronomic trait has become a reality.

Development of oxidative stress, or the generation of reactive oxygen species is a common denominator in the mode of action of several environmental stresses including drought and salinity. During the course of evolution, plants have developed a variety of antioxidative defense mechanisms, to protect themselves against the damaging effects of reactive oxygen species. However, the potential of these antioxidant defense mechanism(s) varies from plant to plant. A plant could successfully cope up with the implied stress, if it possesses a strong antioxidative defense system, otherwise it succumbs to the external stress. The crucial point is the breakaway level, at which the cells (or plant) defense potential is overridden by external stress.

Under such a scenario, strengthening the antioxidative defense mechanism(s) of a plant seems to be a promising strategy for developing stress tolerant plants.

Ascorbate peroxidase (APX) is one of the most important and widely distributed antioxidant enzymes in plant cells. It is an integral part of Ascorbate-Glutathione cycle and the primary H_2O_2 scavenging enzyme in chloroplasts as well as cytosol of plant cells. In the present project we have tried to overexpress cytosolic ascorbate peroxidase enzyme in *Brassica juncea* (*var*. pusa jaikisan), so as to strengthens the antioxidative defense system of the host plant and to assess the role of ascorbate peroxidase in imparting tolerance to salinity stress.

Hypocotyls obtained from 6-day-old *Brassica juncea* seedlings were used as explants for standardizing the regeneration protocol and for developing an efficient and reproducible transformation system for transforming the host genome with *apx1* gene construct, through *Agrobacterium* mediated genetic transformation. The putative transformants, developed in the first phase were confirmed through biochemical and molecular tests and the transgenic lines/plants were then tested for their salinity stress tolerance potential, using standardized leaf disc assays.

In the initial experiments, *Brassica juncea* hypocotyl explants were transformed with *apx1* gene. The transformed hypocotyls were selected using 30 mg/L Kanamycin as selective antibiotic. The selected hypocotyls were induced to initiate shoot development on MS media supplemented with 2.5 mg/L BAP and 1.0 mg/L. The induced shoots were multiplied on multiple shoot induction media and the healthy shoots of about 3 cm height were transferred to root induction media. Profuse rooting was induced in semi-solid MS media supplemented with 0.5 mg/L IBA. Transformed plantlets were successfully hardened in growth chambers and were later transferred

to transgenic glass house. The successful integration of *apx1* gene insert was confirmed through PCR using *nptII* and *apx1* cDNA primers. The morphological and physiological status of transgenic plants was compared with the wild type plants, using various physiological parameters like Photosynthetic activity, stomatal conductance, chlorophyll fluorescence and SPAD values. The results indicate that the physiological and metabolic status of the transgenic plant was comparable with the wild type plant. The growth profile, flowering and seed setting in transgenic plants were found to be similar to the wild type plants. This study confirmed that the ectopic insertion of *apx1* cDNA into the host genome did not bring about any major alteration in the physiology of the transgenic plants.

The stress tolerance potential of the transgenic plant was evaluated through leaf disc assay using standardized biochemical parameters. The transgenic plants decisively performed better under 200 mM NaCl stress than the wild type plant; as revealed through greater Proline accumulation, increased CS index, higher activities of Ascorbate peroxidase, Guaiacol peroxidase & Total peroxidase and lower H_2O_2 level and lesser membrane damage as measured through MDA contents.

Exposure to 200mM NaCl stress caused greater accumulation of proline in the leaf segments of transgenic plants as compared to the wild type ones. As free proline is known to impart tolerance to reactive oxygen species, a higher level of free proline in transgenic leaf segments indicates better tolerance of this genotype. The extent of membrane damage, as indicated through Malondialdehyde contents, was decisively lower in the leaf segments of transgenic plants. Similar observations came forth from the chlorophyll stability index studies, which showed better statistics for transgenic leaf segments. This clearly shows that transgenic plants posses better ability to detoxify

the potentially damaging reactive oxygen species and have experience lesser oxidative stress. Expectedly, the H_2O_2 contents were also lower in transgenic leaf segments as compared to wild type leaf segments under 200 mM NaCl stress. A higher ascorbate peroxidase activity in transgenic leaf segments caused due to over-expression of *apx1* gene is responsible for the observed levels of H_2O_2; as it is the substrate for the antioxidant enzyme and APX is primarily involved in the scavenging of H_2O_2. Hydrogen peroxide is an extremely toxic reactive oxygen species, under stress, as it can cross the biological membrane and can damage cellular organelles. Therefore, a lower H_2O_2 content in transgenic leaf segments under stress is an extremely important adaptive measure for salinity stress tolerance.

Chlorophyll parameters constitute an array of important indicators of plant health and metabolism. A lower chlorophyll a/b ratio and a higher total chlorophyll content as recorded in transgenic leaf segments, signifies better photosynthetic capacity of transgenic plants under salinity stress. Chlorophyll concentration can be used as a sensitive indicator of the cellular metabolic status; thus, its decrease signifies toxicity in tissues due to imposing stress. The reduction in photosynthesis with advancement of the stress may be due to excessive H_2O_2 accumulation that causes oxidative stress. A lesser reduction in total chlorophyll content may be attributed to improve scavenging of H_2O_2 in transgenic leaf segments. In present investigations the lower Chlorophyll a/b ratio, shown by transgenic leaf segments as compared to wild type leaf segments under stress, indicate better adaptability of the transgenic leaf segments.

Interestingly, the activities of other H_2O_2 scavenging antioxidative enzymes like guaiacol peroxidase and total peroxidase also increased in both wild type and transgenic leaf segments subjected to 200 mM NaCl stress, although a higher increase in the

activities of these enzymes was recorded in transgenic leaf segments; which correlates well with better stress tolerance profile of transgenic leaf segments under salinity stress. The recorded increase in total peroxidase and guaiacol peroxidase activity in plants over-expressing cytosolic ascorbate peroxidase indicates the ability of the cellular defense system to co-regulate the expression of different antioxidant enzymes, through an intricate cross talk mechanism. Thus an efficient scavenging of reactive oxygen species could be achieved through the complex but coordinated expression of antioxidative enzymes. The results obtained in the present investigations reveal that for an effective H_2O_2 scavenging system, a coordinated action of various peroxidases is required. Surprisingly, the activity of catalase decreased in transgenic leaf segments under salinity stress; indicating that catalase may not be a part of H_2O_2 scavenging system, under salinity stress; though it may have a role in normal metabolism under control conditions.

Incidentally, a consequential increase in the activities of Guaiacol peroxidase (GPX) and total peroxidase (POD), caused due to the over-expression of cytosolic ascorbate peroxidase, supports the selection of *apx1* gene as the candidate gene, for strengthening the antioxidative defense mechanisms in *Brassica juncea*.

CONCLUSION

In the present studies, a successful attempt has been made to strengthen the antioxidative defense potential of *Brassica juncea* (*var.* pusa jaikisan), through over-expression of cytosolic ascorbate peroxidase gene. The over expressed enzyme could substantially improve the salinity stress tolerance profile of the host plant, as assessed through various physiological, biochemical and molecular parameters. From the present studies it can be confidently concluded

that cytosolic ascorbate peroxidase plays a pivotal role in salinity stress tolerance mechanism of *Brassica juncea*. This observation is further supported by the concerted increase in the activities of other H_2O_2 scavenging enzymes, mediated by the over-expression of cytosolic ascorbate peroxidase; thereby underlining the existence of a coordinated master regulation of various antioxidant genes/enzymes. Unraveling the mechanisms of this coordinated expression of various antioxidant enzymes and the way they cross talk, through different signaling molecules will help us to fully explore and understand the intricacies of cellular antioxidant defense mechanism/pathway.

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VITA

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ABSTRACT

Brassica juncea is a widely distributed and economically important oil seed crop, grown throughout the Indian sub-continent. The production and yield statistics of the crop has recorded wide fluctuations because of several environmental factors. Salinity stress is one of the major constraints limiting the overall yield potential of *Brassica*. An underlying mechanism for reduction in crop yield under salinity stress is the excessive production of reactive oxygen species (ROS) that can potentially damage lipids, nucleic acids and proteins, leading to disruption of essential physiological & biochemical processes. Plants possess a complex antioxidative defense system, comprising of enzymatic and non enzymatic molecules for scavenging these ROS. Ascorbate peroxidase (APX, E.C. 1.11.1.11), an important and widely distributed antioxidant enzyme is a part of Ascorbate –Glutathione pathway in the cell. It plays a crucial role in the detoxification of hydrogen peroxide in plants. The efficient scavenging of ROS is needed for increased tolerance to salinity stress. In the present studies, experiments were conducted to determine whether over-expression of APX could provide protection against salinity stress. Cytosolic ascorbate peroxidase (*apx1*) gene, isolated from *Arabidopsis thaliana*, was chosen as the candidate gene for strengthening the antioxidative defense system of *Brassica juncea*.

An efficient and reproducible protocol for *Agrobacterium* mediated transformation of *Brassica juncea* (*var*. pusa jaikisan) was developed using hypocotyl explants. Transgenic plants over-expressing *apx1* gene were developed and the successful integration of *apx1* gene insert was confirmed through PCR using *nptII* and *apx1* cDNA specific primers. The growth & physiological status of the transgenic plants was assessed using parameters like photosynthetic efficiency, stomatal conductance, chlorophyll fluorescence and SPAD values. The results indicate that the physiological and metabolic status of the transgenic plants was comparable with that of the wild type plants.

Leaf disc assay was performed to evaluate the salinity stress tolerance potential of transgenic plants, using standardized biochemical parameters. Under 200 mM NaCl stress, the transgenic plants decisively performed better than the wild type plants; as revealed through greater Proline accumulation, increased Chlorophyll stability index, lower chlorophyll a/b ratio, higher activities of Ascorbate peroxidase, Guaiacol peroxidase & Total peroxidase along with lower H_2O_2 levels and lesser membrane damage as measured through MDA contents. From the present studies it can be concluded that cytosolic ascorbate peroxidase (*apx1*) can be used to strengthen the anti-oxidative defense system in plants and the transgenic *Brassica juncea* plants developed during the study have improved tolerance against salinity stress.