INTRODUCTION

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In the early seventies, the Green Revolution made a drastic increase in the production of major crops, but as the natural genetic variability has been exploited, there does not seem to be further increase in crop production by Green Revolution. The food demands for the increasing population of the world are growing day by day, but the availability of food per-capita worldwide has not increased since 1936 instead has decreased during the last decade (Sharma, 1997) The world population is expected to reach 7 billion within 25 years and over 10 billion by the year 2050, while agricultural production is growing at the slower rate of about 1.8% annually (Altman, 1999). Food production to feed this expected large increase in world population in the 21st century in both developed and developing countries cannot depend solely on conventional methods of crop production. Especially crop and forests cannot be maintained as environmentally protected agriculture. Their survival and continuously increasing productivity, depends upon classical and new breeding methods (Meiri and Altman, 1998).

One of the most important causes of concern for crop improvement is the global trend of per-capita decrease of land. Land the free gift of nature, for agriculture, is under deterioration due to the faulty agricultural practices as well as environmental disasters. This degradation indicates towards decline in production potential of soil, which encompasses fertility level as well as physical, chemical and biological condition of soil. Up to 1950, the size of areas under cultivation increased at about the same rate as that of world population, but from 1950 the rate of increase in agricultural land declined below the population growth (Sharma, 1998). Even under natural condition of vegetation, nature takes 100-400 years or more to generate 10 millimeters of topsoil and 3,000 to 12,000 years to generate enough soil to be sufficient for food and fibre production (Peccei, 1981). About 12% of the world's land surface is used to grow crops and the agricultural area required to support food production, which was 0.44 ha/capita in 1961 and is expected to reduce to 0.15 ha/capita by 2050 (Altman, 1999). So, the next challenge, for plant scientists is to meet the ever-growing needs for food, fibre and fuel for the increasing world population.

To improve the crop production, it is essential to extend crop harvest beyond the traditional regional seasons and utilize the unused land which was not suitable for agriculture. New technologies are required to provide improved plants and crops for growing under unfavourable environmental conditions, such as saline or arid lands.

By the year 2020, more than 90% of world's population would live in the developing countries, under conditions of extreme poverty and forced hunger (Khurana, 1998) and only rapid technological developments would enable the production of larger yields by breaking traditional barriers.

Biotechnology is one of the key technologies to solve this problem. Biotechnology is a rapidly developing area of contemporary science which can change the face of the world with new ideas, improved tools and novel approaches for resolving some persistent, seemingly intractable problems, particularly in food production (Chadha, 1998). This is the technology which can bring improvements in the availability of food, animal feed, renewable raw materials and enhancement of environmental protection and the world will enjoy the maximum benefits from this technology if it is applied safely (Marguard and Ruchirawat, 1995). The Green Revolution of 1970's failed to maintain an increased productivity and also had some serious shortcomings such as the rapid breakdown of pest and disease resistance (Brady, 1995) and a massive loss of genetic diversity with continuing implications for global future food security (Hawatin, 1996). Whereas, biotechnology is the substitute for a proper developmental policy, which has, direct application in genetic improvement and production of crops. New strategies in breeding for disease resistance and the cultivation of resistant varieties have gained precedence over the traditional use of agrochemicals and pesticides (Narayanswamy, 1994).

The myth that more science and more technology will lead to more public welfare (Guston, 1996), needs to be focussed in crop biotechnology (Harris, 1998). Technologies of plant biotechnology are not only to improve the agricultural production, but also for the value addition of ornamental, medicinal and tree species. Biotechnology is now being successfully used to produce crop plants with improved agronomic traits (Ignacimuthu, 1996).

Plant biotechnology offers the most potent emerging techniques for the induction of genetic variability and has far reaching implications in agricultural research and crop improvement programmes (Bajaj and Gosal, 1986). None of the other techniques has made such a profound impact, as plant biotechnology, in translating the breakthroughs of the laboratory into products for the consumer (Prasad and Pareek, 1996) and has led to a major revolution in the culture and propagation of plants.

In recent years, developments in biotechnological techniques have improved not only the agricultural production, but also their nutritional qualities, though these depend on land, water and energy resources, which are usually limited, even then the production and quality has increased many times.

Innovative approaches need to be incorporated for the success of crop improvement programmes, e.g. high yielding and disease-resistant cultivars. Classical breeding methods take long time to introduce disease-resistant varieties, while biotechnology combined with classical breeding procedures would serve to attain the precise breeding goals envisaged at the commencement (Narayanswamy, 1994) and it will open new vistas to enable the early release of improved cultivars and the conservation of germplasm (Bajaj and Gosal, 1986). Thus, biotechnology will undoubtedly complement the conventional methods in generating genetic diversity. Biotechnology promises new techniques for sustainable development and utilization of the natural resources.

Recent advances in plant biotechnology suggest that these techniques represent a potential avenue for accelerating and expanding the development of appropriate technologies for food production in the developing world (Buttel *et al*, 1985) and it has a unique strategic significance in that its development can contribute considerably to the quality of life by providing solutions to plant survival problems.

A number of research laboratories and private industries all over the world, are now actively engaged in obtaining increased food production, higher nutritional value, greater plant resistance to adverse weather conditions, pathogenic agents and pest, through biotechnological approaches. Plant Tissue Culture is an essential component of

Biotechnology, which has contributed tremendously to crop improvement and has a great potential for the future.

In most of the crops, the availability of planting material is the main limiting factor. Though most of them can be multiplied by using conventional methods, some of them do not have any known form of vegetative propagation, e.g. coconut and oil palm, even the cases where traditional methods work, the rate of multiplication is either slow or limited by the non-availability of the scion material from the initial elite stock (Prasad and Pareek, 1996). Thus, advanced technological approaches, like micropropagation and tissue culture can increase the rate of clonal multiplication of such plants into millions. Plant tissue culture has been viewed as a key technology for enhancing the capability for production of large quantities of planting material of selected elite high yielding varieties so as to boost production and productivity (Sharma, 2000). Day by day tissue culture techniques are being exploited for production of elite planting material with desirable characteristics.

In the case of root and tuber crops, continuous vegetative propagation often results in deterioration in quality of planting material. Moreover, improper selection of planting material leads to perpetuation of viral, bacterial or fungal pathogens through clonal generations, resulting in severe crop loss. Thus, production of healthy planting material and their rapid multiplication is necessary for the management of genetic resources of tuber crops. Such requirements have led to the utilization of micropropagation and tissue culture techniques in case of many root and tuber crops (Unnikrishnan and Sheela, 2000).

Micropropagation has many advantages over traditional plant propagation. The main advantages are attributed to the potential of combining rapid large scale propagation of new genotypes, the use of small amount of elite stock and generation of pathogen-free propagules. Plant propagation by tissue culture is also used to develop high quality clonal plant with unique horticultural traits, pest resistance, crop quality or sustainability for environmental stress conditions.

Tissue culture method also provides an ideal method for germplasm storage and for the international distribution of disease-free stocks. It also allows the rapid clonal

propagation of large number of plantlets in a short time and the conservation of germplasm under controlled conditions, which require less space and labour.

Most of the dicotyledonous annuals and foliage plants have been routinely cultured or micropropagated by this technique and over 1000 species of plants have been reported to regenerate *In vitro* (Brown and Thorpe, 1986). Even forests tree species are also increasingly being regenerated by tissue cultures (Hassing, 1989).

Micropropagation is a valuable technique for the eradication of virus from diseased clones of vegetatively propagated crops. During the 1960s and 1970s micropropagation was of greatest economic value in the production of virus-free plants from infected clonal material. Now it has commercial importance in the rapid multiplication of many vegetable crops and selected germplasm.

Potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum* L.) is one of the most important food crops of the world after wheat, rice and maize. Potato production represents half of the world's annual output of all root and tuber crops. The dry matter production of potatoes per unit area exceeds that of wheat, barley and maize (Salunkhe, 1985). Potato accounts for the largest area under cultivation and is the highly consumed single vegetable of the world (Anonymous, 2000). More than 1 billion people consume potatoes worldwide and it is the diet of half a billion people in the developing countries. Potato is an important food crop with wide adaptability and potential to fill in the gap between the food needs and the cereals (Khurana, 1998).

There are seven species of cultivated potato, including diploids (*Solanum anumajanhiuri*, *S goniocalyx*, *S phurıza*, *S. x juzepczukii*); tetraploids (*S tuberosum* ssp. *tuberosum*, *S tuberosum* ssp. *andigena*) and one pentaploid (*S. x curtıolobum*) [Hawkes, 1978b].

Solanum tuberosum ssp., *tuberosum* is the potato cultivated in the Northern Hemisphere and is widely held that ssp. *tuberosum* evolved from ssp. *andıgena* through artificial selection after the later was introduced into Europe in the late 16th century (Miller and Lipschutz, 1984).

The origin of this crop is known to be the highland of the Andes in South America. It is now cultivated in most of the temperate and tropical zones allover the world. The Portuguese traders brought this crop to Indian subcontinent in 16th century. North America and European countries are the largest producers of potatoes, but developing countries are now increasing their production day by day.

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae. The family which also includes other economically important genera, such as tomato, tobacco, egg plant and others. Out of several hundred species of *Solanum*, only potato (*S tuberosum*) and a few others bear tubers (Salunkhe, 1989)

Potato is a tetraploid, dicotyledonous plant and as it propagates vegetatively, there are several problems for its breeding. These include a high level of heterozygosity, the common occurrence of pollen sterility, selection difficulties in the seedlings and difficulties in the germplasm storage and transport and the build-up of viruses (Howard, 1978).

Potato tubers are the underground stem modification for reserve food storage. Although they are rich in carbohydrates, they also provide, significant quantities of other nutrients, such as proteins, minerals (iron) and vitamins etc. At least 12 essential vitamins and minerals including an extremely high density of vitamin-C are available to human body from potato (Amirato *et al*, 1983).

Conventionally, it is propagated vegetatively through tubers, where disease can be easily transmitted to the next generation especially viral diseases, which can lead to a significant decrease in the production. Potatoes are infected by 30 viruses (Khurana, 1992). Once a potato clone is infected by a virus, the disease gets perpetuated and amplified during successive clonal generations, thus seriously affecting the yield potential of the resulting crop.

In the agricultural point of view, the production or yield of a crop can fall dramatically as a result of viral infection and render that particular variety no longer saleable or commercially viable (De, 1992). Thus, based on the observation that the extreme apex of a shoot is free from viruses (Moral and Martin, 1952), meristem culture was the first biotechnological approach, successfully employed to obtain virus-free potato clones (Sarkar and Naik, 2000).

Potato production relies, now a days, on the use of large quantities of toxic chemical pesticides to ensure stable yields (Gaislain and Golmirzail, 2000), which are harmful for the environment as well as for the users, who are directly in contact with these toxic compounds. Reducing pesticides use in potato production is important not only for health and environmental concerns but also to reduce the production cost. Low input, sustainable and environment-friendly agro-techniques, which reduce dependence on chemical fertilizers, toxic chemicals and systemic insecticides etc. need to be developed.

Availability of quality potato seed is another major constraint in potato production, where the cost of seed potatoes alone accounts for 40-60% of the total production cost in many countries (Sawyer, 1979; Wattimena, 1983; Shekhawat *et.al.*, 1997). The conventional breeding of potato involves selection, crossing programmes for recombination and mutation (Bajaj, 1986), which takes a long time to select new varieties and the efficiency of selection is also limited. Thus, starting with 1,00000 seedlings it would take 6-8 years to select a better variety (Wenzel, 1980).

In vitro culture of Potato

Conventional potato breeding is considered very difficult, since, the number of traits that need to be taken care by the breeders are considerably high and is characterized by low multiplication rate and progressive accumulation of degenerative viral diseases during clonal multiplication, whereas, advance tissue culture techniques especially micropropagation, have facilitated the production and multiplication of disease-free potato clones within a limited time and space (Naik *et.al.*, 1998). The first successful establishment of tissue cultures from tuber crop was reported as early as 1951 (Steward and Caplin, 1951). *In vitro* propagation of potato by culture of axillary buds in individual nodes has been used for the rapid multiplication to obtain healthy tubers to be used as seeds (Goodwin *et.al.*, 1980; Hussey and Stacey, 1981; Miller and Lipchutz, 1984; Ranelli *et.al.*, 1988).

The developments in the field of *In vitro* technology have far reaching implications in making potato farming more efficient, profitable and environmentally safe (Chandra, 2000). By using improved media, shoot tip culture had been developed

for the propagation of potato and used as a tool for the production of nuclear potato seed stocks. Shoot tip culture or meristem culture has all the advantages of establishing pathogen-free plants and breaking the cycle of tuber born diseases (Chandra and Upaddahya, 2000). This technique is based on the principal that virus concentration decreases from older to younger tissues of the infected plant (Khurana, 2000). Plants regenerated from meristem tip culture are genetically stable and yield true to type plants (Pennazio, 1971; Quak, 1972; Dhingra *et al*, 1992; Khurana *et al*, 1996). Thus, the ability to free potato of systemic pathogens via meristem tip culture, chemotherapy and thermo-therapy permitted the continuous use of pathogen-free seed stocks and reduced virus infection in the field (Chandra and Upaddahya, 2000).

Microtuber production

Another alternative method over the micropropagation of the potato plantlets is the production of microtubers, produced by allowing *In vitro* plantlets to grow under tuber inducing conditions. Microtubers are particularly convenient for handling, storage and transport of germplasm. Also, unlike *In vitro* plants they do not require any hardening period (Ranelli *et.al.*, 1994). Wang and Hu (1982) were the first to report *In vitro* mass tuberization in potato and successful integration of this technology into production of virus-free seed potato in Taiwan (Naik and Sarkar, 2000). Since then efforts were made to devise effective means of large scale microtuber production and their storage (Wang and Hu, 1982; Estrada *et.al*, 1986; Garner and Blake, 1989; Chandra *et al*, 1992; Naik and Sarkar, 1997), to develop strategies for successful integration of this technology to seed potato production programmes (Ranelli *et al*, 1989; Singh *et al.*, 1994), especially in countries where high quality seed tubers cannot be produced, because there are no vector free areas (Ranelli *et.al.*, 1989, 1990).

Thus, microtubers could become an integral component of healthy seed tuber production programmes, particularly in hot, humid tropical countries (Van der Zaag, 1990). It can reduce the number of field multiplication and increase the flexibility of seed production, improve the healthy status of the ultimate commercial seed produce (Lommen and Struik, 1994).

Potato is a pioneering example where *In vitro* approach has been most successfully adopted for germplasm movement across the world. The International Potato Center (CIP), Lima, Peru, has played a leading role in development of *in vitro* based technologies for exchange and distribution of potato germplasm worldwide (Naik and Sarkar, 2000).

Despite all the care, survival of the microplants is greatly reduced if they are kept in the dark for long period during transit (Estrada *et.al*, 1986), while microtubers have the advantage of being more robust and of surviving longer in darkness. They also offer phytosanitary benefits over the *in vitro* microplants for export without the technical problems associated with transport of the green plantlets. (Naik and Sarkar, 2000).

Factors affecting Microtuber production

Factors that affect microtuber production *in vitro*, include growth regulators, cultivar, light quality, photoperiod, temperature and sucrose concentration. Enhanced microtuber production has been achieved by several workers, using different combinations of plant growth regulators, such as BAP and CCC (Yamamoto and Nakata, 1997; Wang and Hu, 1982; Hussey and Stacey, 1984; Abott and Belcher, 1986), Kinetin (Palmer and Barker, 1973) and medium devoid of any plant growth regulators (Garner and Blake, 1989).

Many other substances have also shown to affect induction of microtubers, such as, abscisic acid (Hussey and Stacey, 1984); the antigibberellin compound (CCC) (Tovar *et.al.*, 1985); auxins such as, NAA (Morozova and Sarkisov, 1978); Jasmonic acid (Pelacho *et.al*, 1991); coumarins (Stalkmecht and Farnsworth, 1982); triazols (Chandra *et al.*, 1992).

The influence of different light spectra on the growth and development of meristem tips of potato has been reported by Pennazio and Redolfi (1973). Cool, white fluorescent light is suitable for fostering optimum growth in potato micropropagation. Pelacho and Mingo-castel (1991), reported the effects of photoperiod on *in vitro* tuberization of potato. They have found that increase in the photoperiod inhibit tuberization in stolon cultures of potato.

Microtubers induced in continuous darkness are white, whereas those induced under day-night (light-dark) photoperiod are green (Gopal and Minocha, 1997). White microtubers exhibited a better post-harvest performance in the field with respect to number of nodes, leaflet size, especially the width; uniformity of minitubers, tuber colour, weight etc.

Sucrose is also a major factor, affecting tuberization, as it is the effective source of carbon and energy for *in vitro* tuberization of potato (Mes and Menge, 1954). An increase in sucrose concentration from three to eight percent induced early tuberization (Abott and Belcher, 1986; Hussey and Staccy, 1984), but a sucrose level above it was inhibitory (Van Handel, 1968; Garner and Blake, 1989). According to Sarkar and Naik (1997), there is no quantitative relation between sucrose absorption and reducing sugar appearance. Increased sucrose utilization resulted in an increase in the weight and yield of the microtubers, but did not affect the number of microtuber production. This showed that sucrose was responsible for increase in the weight of microtubers rather than their induction.

The optimum temperature for microtuberization is 15°C – 20°C. Temperature below 12°C and over 28°C is strongly inhibitory (Okazawa, 1967; Wang & Hu, 1982). But Wattimena (1983) and Thiome and Pett (1982) obtained microtubers at lower temperatures (8°C-10°C). This may be due to the absence of growth regulators, especially cytokinin in the microtuber induction medium (Wang and Hu, 1985).

Although the optimum conditions for formation of microtubers vary among different reported studies, it has been widely accepted that the formation of microtuber is promoted by cytokinins (e.g. Zeatin, Kinetin or BAP), in the media. Gibberellins promote stolon elongation, but inhibit tuber formation (Okazawa, 1959), whereas growth inhibitors have a contradictory effect. Ethylene influenced stolon growth and seemed to have a role in the maintenance of the diageotropic growth habit characteristics of stolons.

During microtuber formation, reserve food from plantlets are partitioned to microtubers. Therefore, the initial availability of reserves in the plantlets determines the production potential of microtubers *in vitro* (Garner and Blake, 1989).

One of the major limitations of the microtubers is their small size. Small microtubers are more vulnerable to storage damage (Naik and Sarker, 1997) and difficult for direct field planting (Jones, 1988). For commercial seed potato production microtuber size is more important than microtuber number.

Microtuber Development

Potato crop is usually vegetatively propagated through field produced seed tubers. This field operation may be preceded by a myriad of different vegetative *in vitro* and *in vivo* methods of multiplication, many of them include a step in which tuberization takes place. Therefore, understanding the regulation of tuber formation is very crucial. Detailed knowledge on the timing of tuber initiation is also required to understand the formation of tubers.

But it is not easy to predict exactly when tuberization will occur or which stolon tips will tuberize. By the time stolon tips have undergone visible swelling, initial changes have already occurred. Single node cuttings from induced plants provide a much more predictable system to study these events (Gregory, 1956; Ewing and Waring, 1978, Ewing, 1981).

The formation of potato tubers comprises two different aspects; (a) the morphological development of the tuber and (b) the biochemical changes resulting in the formation and storage of starch. During the development of tubers, number and size of parenchymatous cells in pith, cortex and perimedullary tissues increase (Artehwages, 1918; 1924; Leshem and Clowes, 1972; Hayward, 1938; Reeve *et al*, 1969; Cutter, 1978; Peterson et al. 1981). The larger size of *in vivo* tuber is due to further development of the perimedullary region which is lacking in *in vitro* conditions.

Minitubers

Microtubers can be directly transplanted in a greenhouse or nethouse. Plants from microtubers have fewer main stem than those from conventional tubers (Wattimena et al., 1983; Haverkort *et al*, 1991). Tubers obtained from these plants (minitubers) are smaller than the normal seed tubers but larger than microtubers. Minitubers are relatively easy to manage (Espinozoa *et.al*, 1984; Hussey and Stacey,

1981) and serve as an initial source of prebasic seed stock for the certified seed potato industry (Metching *et al*, 1993; Vodenik and Jenke, 1992). Minitubers can minimize the risk of virus contamination of seed tubers.

The usefulness of microtubers and minitubers depends upon the performance of crops raised from them. Haverkort et al (1991) have reported that physiologically older microtubers performed better than younger microtubers. Tuber yield from plants, grown from smaller microtubers is usually lower than that from larger ones (Tover *et.al.*, 1985; Alsadon *et.al.*, 1988).

Encapsulation of potato nodal segments

Synthetic seed technology forms an ideal system for propagation, conservation and exchange of plant material (Redenbangh, 1990). In crop species, where somatic embryogenesis is not common, attempts were made to encapsulate i*n vitro* derived vegetative propagules in a appropriate gel and to use the encapsulated segments as synthetic or artificial seeds (Bapat, 1993; Piccioni and Standerdi, 1995). Single node cuttings from micropropagated potato plantlets can be encapsulated in a synthetic matrix with calcium chloride and sodium alginate. This alginate coating serves as endosperm, consisting of carbon sources, nutrients, growth regulators, antimicrobial agents etc.

Advantages of micropropagation and microtuberization of potato (*Solanum tuberosum* L.)

1. a large number of propagules can be obtained from a single plant
2. no seasonal constraints
3. a constant flow of disease-free plants is possible from the original i*n vitro* stock developed through meristem culture
4. needs less space and short time span
5. conservation or prolonged maintenance of i*n vitro* materials provides an effective system for establishing germplasm collection
6. it has facilitated international exchange and distribution of clonal material in the disease free condition

7. a high degree of stability of i*n vitro* plant material, with practical storage periods cryogenic storage at ultra-low temperature in liquid nitrogen
8. small dormant microtubers are convenient for handling, storage and distribution
9. microtubers do not need the time consuming hardening period in greenhouse and can be adopted easily to large scale mechanized planting in the field
10. microtubers and minitubers, can be used in seed production programmes to reduce the number of field multiplication, which can increase the flexibility of seed production, improve the health status of the ultimate commercial seed produce and reduce the time required for adequate volumes of seed from new cultivar to become available.

Objectives:

Considering all the above advantages of tissue culture techniques, current research work was undertaken with a view to:

1. Standardizing a protocol for i*n vitro* establishment of isolated meristem of var Dheera and var Heera
2. Standardizing a protocol for shoot multiplication
3. Standardizing conditions for optimum production of microtubers
4. Understanding the development of microtubers
5. Understanding the histochemical changes during microtuberization viz. changes in the levels of metabolites such as carbohydrates
6. Standardizing the storage condition, shelf life and germination of microtubers
7. Producing synthetic seeds of potato using nodal segments and standardizing the condition for their storage and germination
8. Producing minitubers from microtubers