CHAPTER V

DISCUSSION

In recent years, methods of tissue culture *in vitro*, which is used to provide rapid vegetative propagation of herbaceous plants, has also been applied successfully to woody species. In the beginning tissue culture of trees progressed with little success. Majority of reports earlier revealed only a low or sporadic production of plants and were usually related to seed or seedling material (Winton, 1978).

Choice of explant, age of the plant, type of hormones play a key role in *in vitro* propagation of trees. Methods of plant propagation by *in vitro* methods include shoot culture with proliferation of axillary or adventitious shoots and callus culture with regeneration of shoots or embroyoids (Hussey, 1978).

In the present investigation nodal explants along with axillary buds were used as the source material. Shoot culture (shoot tips or buds) is often sufficiently reliable with herbaceous plants to be used commercially (Holdgate, 1977). Oka and Ohyama (1975) performed experiments in order to know the suitability of type of

material for in vitro propagation of mulberry. They used three kinds of explants and various aged axillary buds for their study. Young greenish buds with long or short stem grew into leafy shoots on MS + NAA or MS + NAA + BA medium respectively. Excised greenish buds did not grow in var. Ichinose, but they grow in var. Kenmochi when supplemented with BA. Excised brownish buds that were more aged than greenish buds did not show any growth in both varieties.

The physiological status of the explant has been of prime importance with adult trees. Extensive studies at the Association Forest - Cellulose (AFOCEL) research institute in France indicated that explants from adult forest trees were frequently slow to commence growth in vitro, or failed completely unless selected from rejuvenated tissues. When natural rejuvenation is absent, it has been induced by various treatments such as grafting, shoot pruning, maintenance of high fertiliser levels, vegetative propagation or spraying with cytokinins (Franclet, 1979). Two months after pruning, young rejuvenated twigs up to 5th node were collected in the present study. The response of explants varied depending upon the variety and type of medium used.

The choice of a particular medium depends mainly on the species of the plant, the tissue or organ to be cultured. In the present investigation, B5 and MS media were tested for callus initiation in Mysore local and Kanva-2, days required for callus initiation and per cent frequency of callus in Kanva-2 and Mysore local differed considerably in both the media. Kanva-2 responded well on MS medium fortified with 2mg/l 2,4-D, than on B5 medium with same supplement. This may be due to high nitrate requirement of Kanva-2 and nitrate is relatively high in MS medium when compared to B5 medium. The present study clearly demonstrates that there will be 100% response in Kanva-2 when MS medium was used whereas Mysore local can proliferate better (60%) on B5 medium.

Callus was yellow and smooth in Mysore local, nodular and yellow in Kanva-2. During subsequent cultures callus ceased to grow and appeared brown in colour due to accumulation of phenolic compounds. This was reported by Tewary et al., (1989) in mulberry and also in other angiospermic taxa by Shah and Mehta (1976) and Singh et al., (1982). By using 0.1% activated charcoal this was prevented in the present investigation. Activated charcoal adsorbs many organic and inorganic molecules from the culture medium (Mattson

and Mark, 1971). Although the precise effects of activated charcoal are unknown, there are several possible modes of operation. It may remove contaminants from agar (Kohlenbach and Wernicke, 1978) and secondary products secreted by the cultured tissues (Wang and Haung, 1976; Fridborg et al., 1978). In addition, some of the positive effects of activated charcoal may be due to darkening of the support matrix and thus approximating more closely soil conditions (Proskauer and Berman, 1970).

For callus initiation, organised development of tissues in culture systems require certain growth regulators such as auxins and cytokinins. Auxin-Cytokinin supplements are instrumental in the regulation of cell division, cell elongation, cell differentation and organ formation (Dodds and Robertes, 1985). The auxins and related growth regulators normally used in the initiation and maintenance of callus cultures are Indo-3yl-acetic acid (IAA), NAA and 2,4-D (10^{-7} -10^{-5} M). Though there were many reports on the use of 2,4-D for callus initiation in Mulberry, comparative studies of two varieties Kanva-2 and Mysore local in two different media (B5 and MS) fortified with 2,4-D and the effect of different of concentrations of 2,4-D on callus intiation was studied for the first time.

By using 10M 2,4-D Oka and Ohyama (1973) obtained two types of calli from stem segments of Mulberry var. Ichinose. As for the effect of auxins better callus formation was observed on medium with 2,4-D than with IAA or NAA. Oka and Ohyama (1976) reported that the addition of 2,4-D remarkably enhanced the callus formation at the lower part of the explants, but the shoots developed rarely. The effect of different concentrations of 2,4-D (0.5, 1, 1.5, 2mg/l) on sprouting, rooting and callus proliferation was studied in the present investigation. High concentration of 2,4-D (2mg/l) showed remarkable suppression of both sprouting and rooting. Gamborg et al. (1976) stated that 2,4-D is a powerful suppressant of organogenesis and it whould not be used in experiments involving root and shoot initiation. Though this herbicide is suppressing organogenesis at a concentration of 2mg/l, however, there was 100% sprouting in Kanva-2 and 50% in Mysore local at a low concentration (0.5 mg/l) of 2,4-D. Rooting from the cut ends can also be seen in both the varieties at this concentration. As the concentration of 2,4-D increased from 0.5 to 1.5mg/l the percent of sprouting and rooting decreased.

At 2mg/l there was no sprouting at all and it was represented in Table-IV. Positive effect of low

concentration of 2,4-D on organogenesis was further confirmed by sub-culturing the explants from 2mg/l 2,4-D containing medium to medium which was fortified with 1mg/l 2,4-D.10 days after sub-culturing 6% explants developed into entire plants in Mysore local. 12% entire plants, 35% rooting, 41% sprouting was recorded in the case of Kanva-2. By these results it can be shown that low concentration of 2,4-D is not suppressant of organogenesis. Sekih et al., (1974) reported highest growth rate of callus obtained with 2,4-D followed by IAA and NAA in the light condition. In our present study highest callus initiation was observed at 2mg/l 2,4-D concentration whereas NAA and IAA induced root formation. The explants cultured on medium without any hormones, sprouted very little and did not show any further growth or callus proliferation. Patel et al., (1983) cultured stem, petiole segments, leaf discs on MS medium alone which has not showed any response, but when supplemented with IAA, IBA, NAA, 2,4-D at 0.5, 1,2 mg/l they proliferated into callus tissue within 4 weeks. Callus tissue was green and friable on 2,4-D medium, whereas on NAA and IAA medium brownish, compact allus was reported. Though the nature of callus in our present study was smooth and yellow in Mysore local, nodular and yellow in Kanva-2 in the beginning, it has become compact and greenish patches

appeared when sub-cultured on to the medium fortified with kinetin and IAA. Tewary et al., (1989) cultured leaf explants of S1 strain of mulberry on BAP and Kinetin, does not provided any result. However, they found that BAP (2 mg/1)+ 2,4-D (0.5 mg/1) was most suitable for callus proliferation and maintenance of callus for long term culture. Many investigators amply stressed the importance of 2,4-D on both callus initiation and proliferation. There is every need to provide considerable interest on the type of media which also proved the significant effect on callus initiation and proliferation. Thus the present study provides a scope of rapid callusing of Kanva-2 on MS + 2,4-D (2mg/1). This in vitro technique for rapid callusing can be exploited for plant regeneration, in Kanva-2 and Mysore local.

Gautheret (1934) noted the formation of "bud massifs" in cambial tissue cultures derived from trees. Such bud formation might be termed spontaneous, but only in the sense that the stimulus was endogenous in nature rather than present as a component of culture medium. Stimulatory chemicals such as adenine (6-aminopurine) and Kinetin (6-furfuryl amino punine) placed in a medium increase the chance of obtaining bud initiation. (Haissing, 1965). Organised development in tissue

culture systems is strongly dependent on the growth regulator balance in the medium (Halperin, 1969; Torrey, 1966). In tobacco callus a basic regulatory mechanisms underlying shoot and root formation involves a balance between auxin and cytokinin (Skoog and Miller, 1957).

Effect of Kinetin, IAA and NAA on bud development and root formation of sub-cultured nodal explants of Kanva-2 and Mysore local was studied. Kinetin considered to be a critical factor in bud formation since it has been termed a "highly effective adenine" by Miller (1961). The presence of cytokinin in the medium seems to be necessary for axillary bud development in many herbaceous species (Hussey, 1980). Mapes et al., (1981) have described a method for inducing axillary buds in the axils of cotyledons of Pseudotsuga menziesii and they found that cytokinin and auxin were a prerequisite for development.

Callus tissues were isolated and sub-cultured on to a fresh media containing one with 2mg/l Kn + 1 mg/l IAA and another with 1 mg/l Kn + 1 mg/l IAA. Shoot regeneration or bud formation in callus was not achieved by using only Kn. But better spouting can be achieved in 2mg/l Kn + 1 mg/l IAA, (30% in Mysore local and 50% in Kanva-2), when compared to 1 mg/l Kn + 1 mg/l IAA

(25% in Mysore local and 1% in Kanva-2) and there was no rooting. After 6 days, along with axillary shoots, development of female inflorescence was observed in Kanva-2. These developed into fruits after 20 days and such observation was not seen in the case of Mysore local. Such inflorescence development can be observed only when cultured on Kn and IAA containing medium and there was no development of inflorescences on 2,4-D or Kn containing medium. Oka and Ohyama (1975) reported rarely developing flowers in mulberry var. Kenmochi and Ichinose. Both male and female inflorescences were reported by Patel et al., (1983) in axillary bud cultures of Mulberry on MS + IBA + Kn.

In the present investigation after the complete development of axillary shoots these were transferred to rooting medium. IAA, NAA and 2,4-D were used and their rooting efficiency was tested. The stimulatory effect of auxins in the root formation depends partly on the type of auxin employed. Hay (1962) has shown that bean and Silene stem pieces initiate more roots when planted on a medium containing 2 - (2,4,5-Trichloro phenoxy Propionic acid (Silvex) rather than 2,4-D or no auxin. 0.1 mg/l NAA could induce roots in Morus alba Var. Yanagiba (Oka and Ohyama, 1980). About 95% of shoots were rooted within 3 weeks of transfer by supplementing

with 0.5 mg/l NAA (Narayan et al., 1989). Vigorous rooting of 35% in Mysore local and 45% in Kanva-2 was observed in medium containing NAA (1 mg/l) after 20 days. Comparatively NAA is more efficient rooting agent than IAA and 2,4-D. After one sub-culture on to the same mdium containing NAA 100% rooting was observed in both the varieties.

Ohyama and Oka (1976), concluded that exogenous supply of BA and NAA is necessary for morphogenesis. Bud cultures involved the entire rudimentary vegetative shoot collected either just before bud break or during preceeding dormant season. The advantage of this method over propogation by callus is that shoot apex is a priori present and does not have to be induced, only root induction being required. The main disadvantage is that one explant will form only one propagule, not the hundreds or thousands that are produced in callus and suspension cultures (Bonga, 1977).

From the present study it can be concluded that the nutritional requirements for getting callus culture and micro propagation can be further exploited for the applied studies and commercial propagation of the two varieties undertaken.