INVITRO SHOOT MULTIPLICATION IN CERTAIN MULBERRY VERITIES OF M₅, V₁, S₃₆ AND ANANTHA

P.Varaprasad¹ and A.Vijaya Bhaskara rao²
¹Department of Sericulture, Sri Krishnadeveraya University, Anantapur-515003, A.P.
²Department of Ecology and Environmental Sciences, Pondicherry Central University, Pondicherry

Corresponding author’s: P.Varaprasad

Abstract
Silk production is expensive; consequently, silk is considered a fiber of luxury. It is thought that silks expense, beauty and hand contributed to the beginning of the manufactured fiber industry. People wanted fabrics that looked and felt like silk but without the cost so they tried to manufacture fibers similar to silk. Auxiliary bud explants were cultured on basal medium (MS) supplemented with various cytokinins, auxins and antioxidants either alone or in combination. BAP, Kinetin and 2-ip were used to select best cytokine for shoot proliferation. BAP was found to be effective than 2-ip and kinetin. BAP of 2mg⁻¹ was found to be optimum concentration in four mulberry varieties. To improve further shoot multiplication rate of various combinations of three cytokinines + auxin were used. Highest shoot multiplication from the auxiliary bud explants of Morus Spp varieties were observed on MS medium with BAP 2 mg⁻¹ +NAA 1mg⁻³.

Keywords: MS medium, BAP, NAA, Morus Spp varieties

I. INTRODUCTION

Propagation of plants through shoot tip culture allows recovery of genetically stable and true-to-type progeny (Barakat and El-Lakany, 1992; Barakat, 2008).The proliferation of auxiliary shoot from cultured shoots apices and nodal segments is greatly influenced by the nature of the culture medium used. Hence, it becomes essential to optimize culture conditions for a particular clone/cultivar/rootstock or newly bred line that needs large scale planting but availability of sufficient planting stock is a limitation. The present study describes an efficient procedure for the mass propagation of the Mulberry varieties and successful establishment of plantlets in the field. This permits the perpetuation of the unique traits of certain strains. Mulberry is one among the recalcitrant species and is highly dependent on the genotype of explant and combination of growth regulators (Sahoo et al.,1997; Bhau and Wakhlu, 2001; Feyissa et al., 2005), as there are variabilities in regeneration among mulberry varieties (Tewary et al., 1996; Bhau and Wakhlu, 2003; Rao et al., 2010), the main aim of study is to develop and standardize a reproducible invitro multiple shoot induction protocols for quick regeneration of economically important mulberry varieties. Safdari and Kazemitabar (2009) studied on two races of purslane; agronomic purslane and wild one, and they exhibited that there are tangible differences between these two types of purslane in response to tissue culture approaches, such that different explants or hormonal treatments were suitable to different aims for each type. Treatment with 2-iP gave chimeric new shoots in 90 days. This is the first report of somaclonal variation in G. jasminoides leaf tissue from explants receiving 2iP. Treatment with 2-iP yielded 100% chimeric plants.
II. MATERIALS AND METHODS

Actively growing plant tissues requires a continuous supply of inorganic chemicals, which constitute the macronutrients and micronutrients. Potassium nitrate was used in combination with ammonium nitrate in MS medium whereas potassium nitrate was used as a single nitrogen source in B5. Phosphate was supplied as sodium dihydrogen phosphate in B5 and potassium dihydrogen phosphate in MS. Calcium chloride was added for the calcium requirement in both media. Concentrated stock solutions of micronutrients listed in the tables were prepared. Iron stock was prepared separately to avoid problem with iron solubility, and it was prepared in a chelated form as the sodium salt of ferric ethylene di-amine tetra acetate. Different concentrations of 2, 4-D, IAA, NAA and Kinetin were used to study the callus initiating abilities and regenerating abilities of explants of mulberry varieties. These growth regulators were dissolved in suitable solvent before preparing stock solution. Thiamine- HCl, Nicotinic acid, pyridoxine HCl were added in both the media. The amount of thiamine was relatively more in B5 medium than in MS medium. Glycine was added to MS medium. 0.1% activated charcoal was supplemented to the nutrient media as it adsorbs secondary products secreted by cultured tissues. 20,000 mg/l sucrose was added for both MS and B5 media. Sterilized double distilled water was employed in all tissue culture media, including the water used during the culture procedure. For semi-solid media, add agar at a final concentration of 6-10 g/litre prior to autoclaving. It is important to use a good quality, bacteriological grade agar for plant cell culture work. The formulation for MS and B5 media approximately 50 ml of double distilled water was taken in a 100 ml beaker. Salts were weighed according to the first column of the table I. weighed salts were dissolved separately. Solution was transferred to the 100 ml of volumetric flask and made up to the mark. This micronutrient stock was stored under refrigeration. To 50 ml of double distilled water, weighed Na2EDTA (according to column 1 in Table I) was added and boiled to dissolve. Weighed FeSO4.7H2O was added to the boiling solution. After 5 minutes the solution was transferred to the volumetric flask of 100 ml capacity. DDH2O was added to make the solution to final volume. Iron stock was stored at room temperature. Vitamins are weighed according to the column 1 of table I and dissolved in 50 ml of DDH2O. This vitamin mixture was transferred to the 100 ml volumetric flask and double distilled water was added to the final volume. Vitamin stock was stored under refrigeration.10 mg of 2, 4-D was dissolved by adding 2-3 drops of ethanol. Few ml of DDH2O was added and then transferred to the volumetric flask. This was made up to 100 ml by adding double distilled water. 10 mg kinetin was dissolved in few drops of 1 N HCl. About 10 ml of DDH2O was added and transferred to the volumetric flask (100 ml). Kinetin was made up to the final volume by adding double distilled water. Indole auxins can be dissolved in 1 N Na OH IAA was dissolved in few drops of 1 N Na OH and this was transferred to a volumetric flask of 100 ml after adding 10 ml of distilled water. DDH2O was added in order to make up the solution to the final volume. NAA can also be dissolved in 1 N Na OH. The same procedure given to the IAA stock was followed to prepare NAA stock. The Hormone stock solutions were stored in refrigerator. All the stock solutions were labeled including the concentration and date of preparation. All the stock solutions were used within 30 days and discarded after 30 days.

III. RESULTS AND DISCUSSION

Auxiliary buds of, mulberry varieties of M5, S36, V1 and Anantha were aseptically inoculated onto MS medium supplemented with different concentrations of cytokinins and auxins either alone or in combinations, the results were presented in tables 5.48 and Figures 5.1 to 5.48. Morphogenesis was noticed in all the explants inoculated onto MS medium fortified with growth regulators as described in materials and methods. Morphogenesis in the form of shoot bud induction was noticed in cytokinin (either 6-BAP or kinetin or 2-iP) alone or high cytokinin to low auxin (IAA, NAA, IBA, Picloram and 2, 4-D) combinations in the medium. Shoot regeneration in percent response at different concentration of
6-BAP were significantly variable. In M$_5$ variety shoot regeneration in percent response significantly increase from the concentration of 0.5 to 3.0 and decreased with increasing concentration of 6-BAP. The order of increase /decrease at different concentrations was 0.5 mg$^{-1}$ 6-BAP < 1.0 mg$^{-1}$ 6-BAP < 2.0 mg$^{-1}$ 6-BAP < 3.0 mg$^{-1}$ 6-BAP < 5.0 mg$^{-1}$ 6-BAP > 10.0 mg$^{-1}$ 6-BAP. A similar trend was also observed in V$_1$, S$_{36}$ and Anantha. The data presented in the showed that the shoot regeneration in percent response was increased significantly with increasing concentration up to 3.0 mg$^{-1}$ of kinetin and the trend was decreased with increasing concentration of kinetin at (10.0 mg$^{-1}$).

A similar trend was also observed in V$_1$, S$_{36}$ and Anantha. However there is no significant variability in percent response of shoot regeneration among the four varieties. Percent response in the M$_5$
at different concentration of 2-ip were presented. The percent response of shoot induction was significantly increased from 0.5 mg⁻¹ to 3.0 mg⁻¹ and the percent response was decreased with increasing concentration of 2-ip. The significant increase was also observed in V₁ variety, S₃⁶ and Anantha the order of increase/decrease at different concentrations was 0.5 mg⁻¹ 2-ip < 1.0 mg⁻¹ 6-BAP < 2.0 mg⁻¹ 6-2-ip < 3.0 mg⁻¹ 2-ip >5.0 mg⁻¹ 2-ip >10.0 mg⁻¹ 2-ip. Further there was no significant variability in percent response of shoot regeneration. From the data in, it is observed that the number of shoots per explants were increase significantly with increasing concentration of 6-BAP up to mg⁻¹ and decreased with the increasing concentration of 6-BAP. The order of increase /decrease at different concentrations was 0.5 mg⁻¹ 6-BAP < 1.0 mg⁻¹ 6-BAP < 2.0 mg⁻¹ 6-BAP < 3.0 mg⁻¹ 6-BAP >5.0 mg⁻¹ 6-BAP >10.0 mg⁻¹ 6-BAP. Further the number of shoots per explants in V₁, S₃⁶ and Anantha also exhibited similar trend with an increase in concentrations of 6-BAP. The estimated No. of shoots per explants in mulberry variety of M₅ at different concentrations of kinetin was presented. The number of shoots per explants were significantly increased with the increased concentration of kinetin up to the concentration 3.0 mg⁻¹, and decreased with the increased concentrations. In V₁, S₃⁶ and Anantha the number of shoots significantly increased till up to the concentration of 3.0 mg⁻¹ of kinetin and the number of shoots decreased with increasing concentration of kinetin. From the data presented in table 21 and figure 21, the number of shoots per explants increased significantly with the increased the concentrations of 2-ip up to 3mg⁻¹. Further there was a decline in number of shoots with increased the concentrations of 2-ip. In V₁ variety, S₃⁶ and Anantha the number of shoots significantly increase till up to the concentrations of 30 mg⁻¹, and the number of shoots decreased with increasing concentration of 2-ip. Shoot regeneration in average shoot length at different concentrations of 6-BAP were significantly variable. In M₅ variety shoot regeneration in average shoot length significantly increased from the concentration of 0.5 to 3.0 and decreased with increasing concentration of 6-BAP. The order of increase /decrease at different concentrations was 0.5 mg⁻¹ 6-BAP < 1.0 mg⁻¹ 6-BAP < 2.0 mg⁻¹ 6-BAP < 3.0 mg⁻¹ 6-BAP >5.0 mg⁻¹ 6-BAP >10.0 mg⁻¹ 6-BAP. A similar trend was also observed in V₁, S₃⁶ and Anantha. The data presented showed that the shoot regeneration in average shoot length was increased significantly with increasing concentration up to 3.0 mg⁻¹ of kinetin and the trend was decreased with increasing concentration of kinetin at (10.0 mg⁻¹). A similar trend was also observed in V₁, S₃⁶ and Anantha. However there is no significant variability in average shoot length of shoot regeneration among the four varieties.

Average shoot length in the M₅ at different concentration of 2-ip was presented. The average shoot length was significantly increased from 0.5 mg⁻¹ to 3.0 mg⁻¹ and the average shoot length was decreased with increasing concentration of 2-ip. The significant increase was also observed in V₁ variety, S₃⁶ and Anantha the order of increase/decrease at different concentrations was 0.5 mg⁻¹ 2-ip < 1.0 mg⁻¹ 6-BAP < 2.0 mg⁻¹ 2-ip < 3.0 mg⁻¹ 2-ip >5.0 mg⁻¹ 2-ip >10.0 mg⁻¹ 2-ip. Further there was no significant variability in average shoot length of shoot regeneration.

From the data presented in figure 5.37, it is seem that the combination with low auxin (1mg⁻¹) with high concentrations of cytokinines (0.5 mg⁻¹ – 10 mg⁻¹ 6-BAP; 0.5 mg⁻¹ – 10 mg⁻¹ kinetin; 0.5 mg⁻¹ – 10 mg⁻¹ 2-ip) caused an increase in percent response with increasing the concentrations of cytokinines up to the concentration of 3mg⁻¹ of 6-BAP, kinetin and 2-ip in M₅ variety. A similar trend was also observed in V₁ (Figure 5.38). S₃⁶ (Figure 5.39) and Anantha (Figure 5.40). From the data presented in table 5.41 and figure 5.41, it is seem that the combination with low auxin (1mg⁻¹) with high concentrations of cytokinines (0.5 mg⁻¹ – 10 mg⁻¹ 6-BAP; 0.5 mg⁻¹ – 10 mg⁻¹ kinetin; 0.5 mg⁻¹ – 10 mg⁻¹ 2-ip) caused an increase in number of shoot per explants with increasing the concentrations of cytokinines up to the concentration of 3mg⁻¹ of 6-BAP, kinetin and 2-ip in M₅ variety. A similar trend was also observed in V₁ (Figure 5.42). S₃⁶ (Figure 5.43) and Anantha (Figure 5.44). From the data presented in figure 5.45, it is seem that the combination with low auxin (1mg⁻¹) with high concentrations of cytokinines (0.5 mg⁻¹ – 10 mg⁻¹ 6-BAP; 0.5 mg⁻¹ – 10 mg⁻¹ kinetin; 0.5 mg⁻¹ – 10 mg⁻¹ 2-ip) caused an
increase in average of shoot length with increasing the concentrations of cytokinines up to the concentration of 3mg⁻¹ of 6-BAP, kinetin and 2-ip in M₅ variety. A similar trend was also observed in V₁ (Table 5.46 and Figure 5.46). S₃₆ (Figure 5.47) and Anantha (Figure 5.48). Auxins and cytokinins are the most important hormones for tissue culture. Cytokinins promote cell division whereas auxins promote both cell division and cell growth. Naturally occurring cytokinins are a large group of structurally related purine derivatives. Of the naturally occurring cytokinins, two have some use in plant tissue culture media zeatin and 2 Plant tissue culture. There are two kinds of cytokinins one is the naturally occurring cytokinins which includes zeatin, 6-Y, Y-dimethyl allyl amino purine (2iP) and adenine. There is another type which is synthetic cytokinins. This group consists of substituted purines i.e. 6-benzyl amino purine (BAP) and 6- furfural amino purine (kinetin) and phenyl ureas such as thidiazuron. The synthetic analogues kinetin and 6-benzylaminopurine (BAP) are therefore used more frequently.
Among the three cytokinins tested in our study in all four mulberry varieties i.e., M₅, V₁, S₃₆, and Anantha, 6-BAP was found to be the most potent than either kinetin or 2-iP in inducing multiple shoot buds either alone or in combination with low auxin (NAA). Induced shoot buds were elongated further to give raise to individual shoots that survived upon their separation and subculture onto fresh MS medium after 2 weeks with same plant growth regulator concentrations and combination. The superiority of 6-BAP over kinetin was reported in Vigna radiata (L.) Wilczek (Anju Gulati et al., 1992 and Monalisa Chandra et al., 1995) and also over kinetin and 2-iP was also reported in Sizigium cumini L. (Usha Yadav et al., 1990), Ficus carica and Plumbago zeylanica by (Kumar et al., 1998; 2004). In the present study also it was observed that 6-BAP at 3 mg l⁻¹ induced highest number of multiple shoots with more length than the other two cytokinins kinetin and 2-iP. Formation of multiple shoots solely depends on the exogenous supply of a cytokinin. Whereas no shoot bud induction was observed in MS basal medium. These results are in accordance with the findings reported in Philodendron (Sreekumar et al., 2001).

Multiple shoot production by 6-BAP at 3mg l⁻¹ was highest in all the mulberry varieties and significantly different from 1 mg l⁻¹ to 3 mg l⁻¹. These results were concretely similar to the findings reported in Vigna radiata by (Monalisa Chandra and Amita, 1995) and in Morus alba.L by (Ignacimuthu et al., 1997), increasing the concentration of each cytokinin, decreasing numbers of multiple shoots, shoot length and also inducing callus at the base of stem. These results were similar to the findings reported previously in Phaseolus vulgaries L (Chandra I. Franklin et al., 1991) Asiatic Vigna species (Rensto A. Avenido et al., 1999) Vigna radiata (L.) Wilczek (Anju Gulati et al., 1990; 1992; 1994), in many plant species 6-BAP was more effective than the other cytokinins, but this was considerably affected by explant type (Vaibhav Tiwari et al., 2001). The results of the present study show that the considerably longer shoots were obtained with 6-BAP than with kinetin. These findings are similar to many other in vitro regeneration systems (Baht et al., 1995). Addition of kinetin in place of 6-BAP in the medium resulted in the production of less number of shoots with stunted growth. These results agree with the results reported in Aogeissus sericea (Rathore et al., 1991; Gurudeep Kaur et al, 1992). However, there was a significant variabilities in different mulberry varieties i.e., M₅, V₁, S₃₆, Anantha.

Different types of cytokinines (6-BAP, Kinetin and 2-ip) in combination with auxins at different concentrations resulted in more enhanced multiple shoot induction, compared with the cytokinins alone in the medium. The cytokinin shows synergetic action with auxin (NAA) at different concentrations to induce multiple shoot production. Results of the present study also showed the synergetic action of different concentrations of cytokinin with auxin to induce multiple shoot induction and elongation. Of these three cytokinins with auxin, 6-BAP (3 mg l⁻¹) with NAA (1mg l⁻¹) induced highest number of multiple shoots and elongation of shoots with higher frequency compared with other cytokinins (kinetin and 2-iP) with NAA (1 mg l⁻¹). 6-BAP along with NAA showed highest percent response for multiple shoot induction after 10 days of inoculation and each shoot attained a length of more than 4.9 ± cms after 4 weeks of incubation. On the whole BAP of 3mg l⁻¹ was found to be optimum concentration in four cultivars. Though there was a similar trend in the entire mulberry varieties in the above said parameters, there were significant percent variations among the mulberry varieties to different combinations of auxins and cytokinines.

IV. CONCLUSION

Auxiliary bud explants were cultured on basal medium (MS) supplemented with various cytokinins, auxins and antioxidants either alone or in combination. BAP, Kinetin and 2-ip were used to select best cytokine for shoot proliferation. BAP was found to be effective than 2-ip and kinetin. BAP of 2mg l⁻¹ was found to be optimum concentration in four mulberry varieties. To improve further shoot multiplication rate of various combinations of three cytokinines + auxin were used. Highest shoot
multiplication from the auxiliary bud explants of Morus Spp varieties were observed on MS medium with BAP 2 mg\(^{-1}\) +NAA 1mg\(^{-1}\).

**ACKNOWLEDGEMENTS**

The first author, P.Varaprasad, gratefully acknowledge the Department of Science and Technology, New Delhi for the financial support through RGNF is thankfully acknowledged

**BIBLIOGRAPHY**


