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Being an important medicinal plant genus *Phyllanthus* has been extensively studied with regards to its different chemical constituents such as lignans (Satyanarayana, 1988; Singh et al., 1989; Sane et al., 1997; Murali et al., 2001; Kale et al., 2001; Kassuya et al., 2005), alkaloids (Mulchandani et al., 1984; Joshi et al., 1986, Tempesta et al., 1988); flavonoids (Hnatysyn 1987), tannins (Ueno et al., 1988), terpenes (Gupta et al., 1999; Youkwan, 2005) and phthalic acid (Singh et al., 1986) etc. In addition, several pharmacological experiments (Thyagarajan et al., 1982; Syamsunder et al., 1985) have also been reported. In spite of many phytochemical and biochemical investigations, there have been few reports on the tissue culture studies in the members of genus *Phyllanthus* viz. *P. amarus, P. fraternus, P. caroliniensis, P. stipulus, P. uriniata* (Under et al., 1991; Rajasubramaniam et al., 1994, 1997; Catapan et al., 2000; Bhattacharya et al., 2001; Catapan et al., 2001; Catapan et al., 2002; Ghanti et al., 2004).

Direct organogenesis

The regeneration protocol standardized in the present study is a reliable system for direct and indirect regeneration of plants from somatic tissues of *P. amarus*. Micropropagation from shoot tip explants as in the present investigation has been achieved earlier by Bhattacharya et al. (2001) using BAP or kinetin alone in concentration ranging from 0.05-5 mg/l or in combinations with IAA at concentrations from 0.05-5 mg/l. Ghanti et al. (2004) reported multiple shoot regeneration with shoot tips, nodal and internodal explants using BAP or kinetin at concentration ranging from 0.5-3 mg/l in combination with 15% coconut milk. Caulogenesis was reported from stem or branch pieces of *P. amarus* in both MS and B5 media in the presence of 2,4-D alone or in combinations of BAP at different ratios. In the present work, shoot tip, nodal and internodal explants were subjected to both MS and B5 medium with different combinations of a wide range of growth regulators for achieving both direct and indirect shoot regeneration. Use of B5 medium in tissue culture of genus *Phyllanthus* was reported earlier by Rajasubramaniam et al. (1997). For shoot multiplication BAP and kinetin were used alone or in combinations. The combination of kinetin and BAP proved to be better. Nielsen et al. (1995) and Tomsone and Gertnere (2003) also reported that media containing two different cytokinins improve the number and the quality of shoots formed as compared to
media with only one cytokinin. Best results for multiple shoot formation, 22 shoots per explant, were obtained with 1mg/l kinetin and 0.5mg/l BAP in B₅ medium. Cytokinins played a predominant role in multiple shoot regeneration of other *Phyllanthus* species, such as *P. carolinensis*, *P. urinaria* and *P. fraternus* (Rajasubramaniam et al., 1997; Catapan, 2000; Catapan, 2002). For other euphorbiaceae species, such as *Excoecaria agallocha* L., cytokinins stimulated shoot proliferation (Rao et al., 1998). The effect is even more pronounced when two types of cytokinins (BAP and Kinetin) were used in combination as shown in the present investigation. Thus synergistic effect of two cytokinins leads leads to enhanced shoot regeneration as reported by Al- Khayri et al. (2001) in *Citrus aurantifolia* (lime) and Ali et al. (2004) in *Gossypium hirsutum* L. In these species, a combination of BAP and kinetin induced higher number of multiple shoots than in any other phytohormone treatments. However correct concentration of combination of growth regulators is necessary as unfavourable concentration may inhibit the growth of cellular mass (Moore, 1984). In our study best shoot elongation was observed at 0.5mg/l BAP +1mg/l Kinetin. Al- Khayri et al. (2001) also reported shoot elongation at 0.25 mg BAP combined with 1mg/l kinetic in lime.

Although there are reports that root initiation is promoted by exogenous auxin (Cho, 1985) and inhibited by exogenous cytokinins (Pierik, 1989). In contrast to these reports, in the present investigation results showed adventitious root formation in medium rich in Cytokinins (1mg/lkinetin +0.5mg/l BAP). In *Kampheria glanga* multiple shoots rooted in medium with BA or BAP + Kinetin (Vicent et al., 1992). Cytokinins are also reported to be effective for adventitious root formation in *Aristolochia bractiota* and other species (Remeshree et al., 1994). These studies support our results of adventitious root formation in *P. amaris* regenerated in the medium supplemented with cytokinins

Indirect organogenesis

The procedure described here is the first successful plant regeneration system for *P. amarus* through indirect organogenesis using stem internodal explant and hypocotyl explant. The present investigation reflects that both MS and B₅ medium supported callus growth. But best callus growth was obtained in B₅ medium. Rajasubramaniam and Pardha Saradhi (1994) also used B₅ medium in *P. fraternus* for induction and growth of callus. The best callus growth in B₅ medium supplemented with different combinations of cytokinin and auxin was also reported in *Cynara cardunculus*, milk thistle and
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*Origanum vulgare* L. by Figueiredo et al. (1987), Kumari and Pardha Saradhi (1992) and Cimino et al. (2006).

The results of the present investigation reflect the variability in callusing response of different explants. Both internodal as well as hypocotyl explants showed callus induction although a faster growing callus was obtained from internodal segments. Stem internodal segments were also reported to be superior in genus *Populus* (Han et al., 2000), mulberry (Jain and Dutta et al., 1992). Variable response for different explant types has also been reported in other species. (Christopher and Rajam, 1996; Arockiasamy and Ignacimuthu, 1998; Pereira et al., 2000; Dhar and Joshi, 2005). Such variation can be attributed to the physiological condition of the explants, which is determined by genetic factors (Baroncelli et al., 1978; Nagarathna et al., 1991).

The age of explants has been reported to influence organogenesis in tissue cultures (Clog et al., 1990; Sharma et al., 1990). The effect of explant age on callus induction potential is in agreement with the responses of *P. fraternus* (Rajasubramaniam and Pardha Saradhi, 1994), *Saussurea obvallata* (DC) (Dhar and Joshi, 2005), sunflower (Peterson and Everett, 1985), bean, pea (Angelini and Allavena, 1989), and Safflower (Nikam and Shitole, 1999). Hypocotyl explants are more sensitive to age of the seedlings compared to internodal segments. It has already been established that younger explants exhibit greater morphogenic potential than older plants (Welander, 1988; Fasolo et al., 1989; Yepes and Aldwincle, 1994), as they might have more metabolically active cells with hormonal and nutritional conditions that are responsible for increased organogenesis (Famiani et al., 1994).

In the present investigation it was observed that 2,4-D and NAA without cytokinin could induce callus, but for better proliferation auxin (2,4-D, NAA) and cytokinin (BAP) were required in combination. This cytokinin –auxin combination has been widely used for callus growth and regeneration in various protocols developed for other species of *Phyllanthus* viz *P. abnormis*, *P. urinaria*, *P. caroliniensis* (Under, 1991), *P. fraternus* (Rajasubramaniam et al., 1994) and other members of euphorbeace family viz *Emblica officinalis* (Sehgal and Khurana, 1985). Under (1991) reported the same ratio for best callus growth although concentration was higher than the concentration used in the present study. Figueiredo et al. (1987) and Cimino et al. (2006) reported best biomass production in *Cynara cardunculus*, milk thistle using BA and 2, 4-D. Kumari and
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Pardha Saradhi (1992) and Jain and Dutta (1992) used 2, 4-D, NAA and BAP individually and in various combinations in *Origanum vulgare* L. and in mulberry. Maximum shoot bud induction and proliferation was obtained in B5 medium consisting of BAP and IAA. BAP and IAA combination was also reported in *Emblica officinalis* for shoot regeneration. Islam and Riazuddin (1993) also used BA (2.0 - 10.0 mg/l) and IAA (0.1 - 1.0 mg/l) for shoot proliferation using callus culture from hypocotyl explants of chickpea. Lawrence and Koundal (2001) reported BAP and IAA as the best combination for production of plantlets from embryonic callus culture of Pigeon pea.

Genetic transformation of *P. amarus*

To obtain a successful transformation system for *P. amarus*, we developed a protocol based on the selection of shoots grown from calli that had developed from *in vitro* grown internodal explant. Most of the shoots we obtained seemed to grow well on selection medium. We however, never obtained a transformed plant from shoot grown directly from the explants. *P. amarus* appears to benefit from a period of callogenesis during which competence for regeneration may be enhanced and transgenic cell can divide sufficiently. This may be attributed to the reason that the non differentiated cell layer in callus mass may help *Agrobacterium* invade the cell. We have observed that preculture along with addition of acetosyringone into the culture medium enhanced the recovery of more kanamycin resistant calli. The initial callus cell layer may have developed during the preculture period and could have provided a place for *Agrobacterium* to penetrate during co-culture. Chemicals such as acetosyringone are recommended in most of crops transformation protocols (Hiei et al, 1994; Ishida et al., 1996; Cheng et al., 1997; Tingay et al., 1997; Zhao et al., 2000; Kumlehn et al., 2006) for vir induction. *In vitro* grown materials are preferred over field grown material because they need no surface sterilization, there by reducing the chances of contamination and have usually less hardiness. Civinova and Sladky (1990) stated the similar reason for use of *in vitro* explants, as they are often more juvenile and less lignified and thus have more regeneration capacity. Southern hybridization pattern of selected To transgenic plants confirmed single as well as multiple gene insertions. Studies have shown that it is desirable to have single gene insertion in transgenic plants, as multiple copies of T-DNA adversely influence the expression of the introduced gene (Stam et al., 1997). There are reports that gene transfer via *Agrobacterium* cause unstable or lack of expression of...
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transgene (Finnegan and Mc Elory, 1994). Further work is in progress in our laboratory to determine the transgene stability and its expression in transgenic lines of \textit{P. amarus}.

Proline, Chlorophyll and Soluble Protein accumulation in transformed \textit{P. amarus}

Research over the past two decades has provided a better understanding of the molecular biology of stress response in plants. Many genes and gene products have been identified which get induced upon exposure of plants to various abiotic stresses—salinity, drought, low and high temperature stress etc (Abdin et al., 2002). Expression and accumulation of a large number of stress proteins in plants exposed to various biotic and abiotic stresses have been proposed to be one of the defense mechanisms of plants (Scrives et al., 1990; Bol et al., 1990; Abdin et al., 2002). Salinity affects plant growth in a number of ways. The first phase of growth response is due to osmotic effect of salt in the soil solution that produces a suite of effects identical to those of water stress caused by drought. Later, there may be additional effects on growth if excessive amount of salt enters the plant.

Results reported in the present study show that transgenic \textit{P. amarus} plants perform better in terms of chlorophyll, soluble protein, and proline accumulation than wild type plants, when exposed to 150-250 mM NaCl and these were in conformity with the results reported by many investigators in transgenic potato, where tobacco osmotin gene was over-expressed (Babu and Bansal, 1998; Babu et al., 1998). Similarly, Xu et al. (1996) produced transgenic rice plants showing higher rate of growth and less damage under drought and high salt (250 mM NaCl) conditions. The difference in the performance between wild and transgenic plants during leaf disc senescence assay of \textit{P. amarus} might have been due to increased membrane permeability under high NaCl concentration (Dhindsa et al., 1981). This suggests that osmotin may be playing some role in enhancing membrane stability, which is evident from the difference in the performance between wild and transgenic plant. The reduction in the plant growth by NaCl involves several mechanisms of growth inhibition including hindrance in nutrient uptake, lowering of water potential and secondary stress such as oxidative stress. Under stress, decline in productivity in plants might be partially at least due to enhanced photorespiration (Sivkumar et al., 2000). Barthakur et al. (2001) also reported over expression of osmotin in tobacco enabled the transgenics to perform significantly better than the wild type plants when subjected to either salt or water stress. They hypothesized that osmotin
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induce substantial increase in free proline accumulation in transgenic tobacco plants with or without stress as compared to wild type plants is most likely the basis of improved performance. Relatively higher proline content was also detected in transgenic tomato plants ecotopically expressing *Arabidopsis* CBFI transcription factor gene (Hseih et al., 2002) and in transgenic *Arabidopsis* over expressing DREB 1A (CBF3) gene (Grilmour et al., 2000). It appears that similar to CBFI and CBF3, osmotin plays a role directly or indirectly in inducing the expression of gene encoding enzymes of proline biosynthesis By activating the proline-5 carboxylate synthetase (P5CS) enzyme that catalyses the rate limiting step in proline biosynthesis expressing the proline catabolic pathway (Igarashi et al., 1997, Savoure et al., 1995; Yoshiba et al., 1995; Kavikishore et al., 1995; Delauney and Verma, 1993) or by suppressing feedback inhibition of proline biosynthesis (Phutela et al., 2000).

As proline is known to protect plants under abiotic stresses by scavenging reactive oxygen species (Alia et al., 1993), it is possible that osmotin-induced proline accumulation is involved in scavenging reactive oxygen species generated during salt and water stress, there by protecting the transgenic *Phyllanthus* plants against these stresses. Proline also acts as a major reservoir of energy and nitrogen, which can be used in resuming growth after stress removal (Chandrasckhar and Sandhyarani, 1996).

Further, it may be pointed out that osmotin is a proline rich protein (Singh et al., 1987) and degradation could also possibly lead to increased accumulation of proline at least under conditions where the protein is over produced (Barthakur et al., 2001). It has been shown in developing grapevine fruits that proline could accumulate in plant cell due to degeneration of proline rich proteins independently and not associated” with either increase in steady state levels of P5CS mRNA or proteins or a decreased in steady state proline dehydrogenase protein (Stines et al., 1999).

In the present observation some reduction in the amount of chlorophyll of the plant *Phyllanthus* was observed while treating with NaCl. The reduction in chlorophyll could be due to interference of Na⁺ and Cl⁻ ions with the enzymes associated with chlorophyll biosynthetic pathway or to a disturbance in the integration of chlorophyll molecules in to stable complex. One of the notable effects of salinity is the degradation of the membrane s of cell organelles (Mitsuya et al., 2000). The change of the thylakoids of the chloroplast has been reported as a typical symptom of oxidative stress which suggests that the damage in the chloroplasts are induced by a photo-oxidative reaction caused by salt stress and is not directly related with salt content in the tissue (Mitsuya et al., 2000).
Comparatively high chlorophyll content suggesting slow rate of senescence in transgenic P. amarus over-expressing osmotin are probably due to the osmotic adjustment effect of proline, and its role in scavenging of reactive oxygen species. Proline has been earlier shown to act as a compatible osmolyte and its increased production confers osmotolerance in transgenic plants (Kavikishore et al., 1995; Nanjo et al., 1999). The results are at par with those obtained by Rehman (2002) in Cichorium intybus. Sarin et al. (2004) also observed that Vigna mungo upon over-expression of the Glyoxalase 1 (Gly 1) gene exhibit tolerance to NaCl and methylglyoxal induced stress and retained more chlorophyll as compared to the untransformed controls under short-term stress conditions. The chlorophyll content (1.65µg/g fresh weight) in transgenic leaf discs were 1.25 times more than that of the control leaf discs (1.32µg/g fresh weight).

Under saline conditions a low water potential enhances the synthesis of ABA (Mizrahi et al., 1970), which plays a crucial role in plant water relations by affecting the movement of solute and water in the tissue (Davies and Mansfield, 1983). ABA also reduces protein synthesis and accelerates protein degradation (Trewavas, 1972). Similarly, osmotic stress and water stress enhances protein degradation and alters the pattern of protein synthesis and accumulation (Dungey and Davies, 1982; Vartanian et al., 1987). The decrease in soluble protein content in both wild type and transgenic P. amarus plants under stress could be due to generation of reactive oxygen species, interrupted transcription and translation, unstable polysomes and enhanced protease activity (Brady et al., 1984; Gibson et al., 1984). Comparatively better performance of transgenic plants of Phyllanthus harbouring osmotin gene even at high salinity may be due to osmotin induced proline accumulation and there by preventing enhanced protease activity, ROS scavenging and high membrane stabilization. More over, the over expression of osmotin gene may have lead to the accumulation of osmotin protein in transgenic plants, accounting for higher protein content in them even under unstressed condition.

Metabolic Engineering and Secondary metabolites

Plants interact with their environment by producing a diverse array of secondary metabolites (Harborne, 1996), which are low molecular-weight organic compounds, usually with unique and complex structures. A range of physiological and ecological functions have been reported for these natural products, such as hormone regulation, organogenesis, plant defense against biotic and abiotic agents, chemical signaling to
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guide pollinators or fruit disperser and plant – microorganism symbiotic interaction (Curir et al., 1990; Van Tunen and Mol, 1991; Vogt et al., 1994; Turlings et al., 1995). Recent research shows that the applications of metabolic engineering are technically possible in plants at the experimental level: Increasing vitamin A content (Ye et al 2000); increasing essential oil production (Mahmoud and Croteau, 2001); decreasing lignin deposition (Abbott et al. 2002); stimulating the bioconversion of secondary metabolites to medicinally important alkaloids (Vander Fits and Memelink, 2000); improving tomato flavor (Wang et al., 2001) and producing biodegradable plastics in plants (Bohmert et al., 2000).

Murphy et al (2002) have identified several treatments that affect the accumulation of phenolics, including osmotic stress by addition of PEG or mannitol to the culture medium and hormone treatments simulating biotic stress (ethylene and methyl jasmonate). These treatments affect accumulation of secondary metabolites differently. Osmotic stress increases the concentration of flavonoids, particularly the anthocyanin. Elicitor like copper sulphate ions are also reported to cause an increase in PAL in palisade cells while phenolic compounds increase in spongy cells, giving rise to necrotic punctuate region in *Phyllanthus tenellus* plants. This elicitor may be a useful tool in the understanding of the regulation of biosynthetic phenolic pathways in *Phyllanthus tenellus* and diversity of plant defense response against plant biotic and abiotic stress (Santiago et al., 2000). There are reports that therapeutically active compounds - phyllanthin and hypophyllanthin in the genus *Phyllanthus* enhanced by certain levels of cadmium due to abiotic stress (Vartica Rai et al., 2005). Based on these reports we expected increase in secondary metabolites in our study since engineered over expression of osmotin gene should lead to the accumulation of osmotin protein, which is a stress protein, but our results are not in agreement with these reports. HPLC results showed no significant differences between wild and transgenic *Phyllanthus* in its phyllanthin content following stress. This may be attributed to the fact that due to enhancement in the level of stress metabolite proline after stress in both wild as well as transgenic *Phyllanthus*, the carbon skeleton and energy needed for the synthesis of other metabolites may be limited.