5. DISCUSSION

The present studies were aimed at assessing the in vitro germination potential of the seeds and regeneration potential of various explants of two therapeutically important species; Solanum xanthocarpum and Malaxis acuminata, and identifying an appropriate chemical stimulus for the purposes. Attempts were made to induce hairy root formation in the two species. Variations in the solasodine producing capability of different parts of in vivo plants, in vitro raised tissues and hairy roots of S. xanthocarpum were also assessed. The results, described in the preceding pages, are discussed as follows in light of the available literature.

5.1 IN VITRO PROPAGATION

5.1.1 Solanum xanthocarpum

5.1.1.1 Seed Germination

In the present study, the seeds of Solanum xanthocarpum at three different stages of maturity (immature green berry, mature yellow berry and fully mature brown berry) were successfully germinated in vitro. Only few attempts have been made earlier to germinate Lycopersicon esculentum (Peres et al., 2001), Petunia axillaris and P. hybrida (Reuveni and Evenor, 2007), Solanum lycocarpum (Pinto et al., 2007), S. platanifolium (Kumar, 1992; Singh, 1990; Singh, 1994), S. surattense (Swamy et al., 2005), and S. torvum (Ripudaman, 1996). Presently, green berried seeds showed maximum percentage of seed germination (84%) compared to the yellow and brown berried seeds; whereas the seeds procured from brown berries showed a consistent germination percentage irrespective of the sucrose concentration in the nutrient medium. Full strength MS medium and medium enriched with higher sucrose concentration (4%) was able to sustain seed germination only in the seeds procured from brown berries. To the best of our knowledge, there have been no reports on such comparative account of in vitro seed germination in the members of Solanaceae.

MS medium was successfully used for seed germination of Solanum xanthocarpum in accord with Swamy et al. (2005) (S. surattense); Peres et al. (2001) (Lycopersicon esculentum); Reuveni and Evenor (2007) (Petunia axillaris and P.
hybrida). Though presently S. xanthocarpum seeds positively responded in full, half and one fourth strength of basal MS medium, half strength MS medium was found to be the best for germination. The use of half strength basal MS medium has been advocated earlier in L. esculentum (Peres et al., 2001), and P. axillaris and P. hybrida (Reuveni and Evenor, 2007). However, Swamy et al. (2005) successfully germinated the seeds of S. surattense on full strength MS basal medium. Singh (1990) and Kumar (1992) germinated the seeds of S. platanifolium in only major elements containing MS medium plus growth regulators.

In the present study, the effect of sucrose was tested at different concentrations of 2, 3 or 4%; the best germination percentage was observed in medium containing 2% sucrose (Fig. 391). Literature studies reveal the use of different concentrations of sucrose for germination i.e. 1.5% in L. esculentum (Zankowski and Rost, 1990) to 2% in L. esculentum (Peres et al., 2001) and seedling maintenance i.e. 3% in S. virginianum (Borgato et al., 2007). In the present study, MS medium was invariably gelled with 0.8% agar. Earlier agar was successfully used at various concentrations i.e. 0.8% [S. virginianum (Borgato et al., 2007)] and 1% [L. esculentum (Zankowski and Rost, 1990)]. All the strengths of MS media and sucrose concentrations were also made in another set of experiments with the presence of AC (0.2%) to test the effect of AC on the germination capability of S. xanthocarpum seeds; AC was generally found to enhance germination in the case of green and brown berry, and ineffective in yellow berry. However, no other reports are available on this aspect in the members of family Solanceae. In the present study, it was found that the seeds, irrespective of their stage of maturity did not germinate under dark conditions. Subsequently, all the seed cultures were maintained under a 16 h photoperiod and 25±2°C temperature regime. A 16 h photoperiod regime was also followed for the germination of S. surattense seeds and they incubated at a temperature of 25±2°C (Swamy et al., 2005). The seeds of L. esculentum were germinated under dark conditions by Zankowski and Rost (1990). After radicle emergence, the Petri plates were transferred to a growth chamber maintained with a 16 h photoperiod (100-160 μ Ein m⁻² s⁻¹ fluorescent and incandescent light). Peres et al. (2001) kept the seeds of L. esculentum in darkness for 4 days until the onset of
germination. Presently, germinated seedlings were maintained in a culture room under 16 h illumination period at 25±2°C for 21 days.

In the present study, radicle protrusion was considered as the time taken for the onset of germination and earliest germination was observed within 7 days. This was followed by greening of cotyledons and opening of cotyledons which took another 8-10 days after radicle protrusion. Similar observations were reported in a study by Pinto et al. (2007) during the seed germination experiments in *Solanum lycocarpum* on a wet sterile Whatman paper. In the present study, seeds of green berries of *S. xanthocarpum* took 7-28 days for germination. Earlier onset of germination was observed in 6-40 [S. *platanifolium* (Singh, 1994)], 6-10 [S. *platanifolium* (Rama Rao, 1996)], 8-15 [S. *platanifolium* (Singh, 1995)], 21-52 [S. *platanifolium*, 32% (Singh, 1990)] days. In the case of yellow and brown berries, onset of seed germination took 1.62-16 and 1.05-8.08 days, respectively in the present study. When two month old (after collection) berries of *S. platanifolium* were used for inoculation, a late germination and poor percentage of germination was observed (Rama Rao, 1996). Ripudaman (1996) reported the initiation of seeds from mature yellow berries of *S. torvum* after 10 days.

In the present studies, only the picked seeds (obtained by using the fruits while still attached to the *in vivo* plant in wild) were used for the experiment and a maximum of 84% germination was observed. This high frequency was observed in the case of seeds obtained from the green berries, while a maximum germination frequency of 73% and 78% was obtained in seeds procured from the yellow and brown berries. However, germination studies of Pinto et al. (2007) in *S. lycocarpum* on wet tissue papers revealed that the seed germination in seeds obtained by picking the fruits from *in vivo* plants (picked seeds) and those obtained from the dispersed fruits (dispersed seeds) was 40% and 90%, respectively. Earlier workers showed a wide range of per cent germination *i.e.* 90% [S. *platanifolium* (Singh, 1990)], 32% [S. *platanifolium* (Kumar, 1992)], 60-90% [S. *platanifolium* (Singh, 1994)], and 76-90% [S. *torvum* (Ripudaman, 1996)].
differentiated cells in cream and green calli, respectively. In the present scenario, it was observed that the different growth regulators promoted the initiation of different types of calli with respect to their colour and consistency (Friable versus compact), even from the same explant. Similarly, Zankowski and Rost (1990) exhibited that each explant callus of *Lycopersicon esculentum* showed differences in consistency, colour and texture. Ritchie *et al.* (1989) reported that the shoot regeneration from callus (leaf explants) of *Beta vulgaris* only occurred from white friable callus and green compact callus could not be induced to regenerate shoots. According to Ritchie *et al.* (1989), cells of white callus were meristematic, while those of green callus were differentiated and so unlikely to produce undifferentiated shoots.

In the current study, it was observed that green coloured compact calli had the maximum chances of showing regeneration and organogenesis (in hypocotyls explants). In a study by Zankowski and Rost (1990) on different explants of *L. esculentum*, the calli failed to exhibit any regenerative potential. Only certain callus types have exhibited regenerative potential in other instances also. A shift was found from shoot formation to embryoid formation being associated with transition from slow growing greenish-white compact callus to fast growing white friable callus (cf. Zankowski and Rost, 1990). Changes in callus morphology on subculturing often indicated endogenous change within the callus and frequent alteration of morphogenetic potential, thereby suggesting that the morphology of callus might act as an early indicator of change within callus (Zankowski and Rost, 1990).

During the course of the current study, only full strength MS medium was utilized for the various explant experiments. Similarly, various other authors have made use of full strength MS medium for regeneration experiments in the members of Solanaceae [Akhond and Bhuiyan (2001); Kang *et al.* (2004); Rama Rao (1996); Rani and Grover (1999); Rani *et al.* (2003); Saito and Nishimura (1994); Singh (1994)]. Bhatia *et al.* (2004) stated that extensive studies needed to be undertaken to determine the effect of nutrient medium other than MS.

In the present study, 3% sucrose was utilized for the various studies on *Solanum xanthocarpm* with good results. Schnapp and Preece (1986) advocated the use of sucrose at a concentration of 30 g l⁻¹ compared to 5.0, 10.0, or 20.0 g l⁻¹ for *Lycopersicon*
Fig. 391 The effect of strength of MS medium, sucrose concentration and stages of seed development on in vitro seed germination in Solanum xanthocarpum

5.1.1.2 Regeneration

In the present study regeneration potential of hypocotyls, cotyledons, roots procured from in vitro seedlings and shoots and leaves procured from in vitro adult plantlets was also assessed on full strength MS medium with and without growth regulators.

Different types of calli were observed; cream coloured, cream-green, cream-green-greyish, fluorescent green, greenish brown, yellow green, and brown coloured callus and sticky cream coloured callus. The brown coloured ones were also friable in nature. The green calli were the most compact ones and organogenic in hypocotyl explants; although cream callus was able to regenerate shoots in the case of leaf explants. Zankowski and Rost (1990) showed the formation of four types of calli; frosty white, frosty green compact, translucent white friable, and translucent brown friable in the different explants of seedlings of Lycopersicon esculentum. Green compact callus, however, was not able to regenerate shoots in the leaf explant cultures. This might be due to difference in types of cells the two types of calli constituted; meristematic and
Discussion

*esculentum* microplant growth. Compton and Veilleux (1988) and Venkatachalam et al. (2000) also used 3% sucrose for their micropropagation studies on *L. esculentum*. Similarly, Gustafon *et al.* (2006) and Kang *et al.* (2004) used 3% sucrose for regeneration studies in *S. tuberosum* and *Scopolia parviflora*, respectively. Sucrose is the preferred carbohydrate source for *in vitro* studies; however, the optimal concentration of sucrose still requires further studies. Earlier, lower concentration of sucrose has been utilized with success [*S. laciniatum* (Herbreteau-Lemonnier *et al.*, 1989; 2%), *Withania somnifera* (Rani and Grover, 1999; 1%)].

Little is known about what constitutes a ‘good’ explant and how the explant is associated with callus formation and subsequent morphogenetic responses. Due to the relation of callus with plant regeneration capacity, characteristics of callus morphology are essential for examining the regeneration response of a specific culture system. Callus formation has been shown to be influenced by the extrinsic (physical and chemical) and intrinsic (biotic) factors. Age effects occurred in responses with juvenile versus adult explant sources; where, typically, younger explant sources formed callus more readily and had greater organogenetic potential. The types of cells constituting the explant also influence the cellular complexity of the callus. The pre-treatment of the explant also influences the morphogenetic potential. The explant location on the parent plant affects the morphogenesis. Keeping these factors in mind, the different explants were tested for their callus forming and/or subsequent regeneration potential in the present study.

### 5.1.1.2.1 Hypocotyl

During the current investigations, hypocotyl segments (1.0 cm long), procured from 15 day old *in vitro* seedlings, and were successfully regenerated on MS medium with and without growth additives. Literature studies reveal that these segments positively responded in *Capsicum* (Gunay and Rao, 1978); *C. annuum* (Valera-Montero and Ochoa-Alejo, 1992); *Solanum laciniatum* (Bhatnagar *et al.*, 2004; *S. platanifolium* (Ripudaman, 1996; Singh, 1990); *S. melongena* (Akhond and Bhuiyan, 2001; Kamat and Rao, 1978); and *Withania somnifera* (Kulkarni *et al.*, 2000; Rani and Grover, 1999; Rani *et al.*, 2003).
Presently, these segments responded via callus and shoot bud generation. Earlier these have regenerated via callus \(C. \text{ annuum}\) (Fári and Czákó, 1981); \(S. \text{ laciniatum}\) \(S. \text{ melongena}\) \(S. \text{ platanifolium}\) (Rama Rao, 1996); (Singh, 1990), shoot bud \(C. \text{ annuum}\) (Gunay and Rao, 1978); \(S. \text{ laciniatum}\) (Bhatnagar \& Czákó, 1981); \(S. \text{ melongena}\) (Kamat and Rao, 1978); \(S. \text{ platanifolium}\) (Rama Rao, 1996); (Singh, 1990), shoot bud \(C. \text{ annuum}\) \(S. \text{ laciniatum}\) \(S. \text{ melongena}\) \(S. \text{ platanifolium}\) (Bhatnagar \& Czákó, 1981); \(S. \text{ platanifolium}\) (Valera-Montero and Ochoa-Alejo, 1992); \(S. \text{ melongena}\) (Akhond and Bhuiyan, 2001); \(W. \text{ somnifera}\) (Kulkarnia \& Czákó, 1999)) and both callus and shoot bud \(S. \text{ laciniatum}\) (Bhatnagar \& Czákó, 1981); \(S. \text{ platanifolium}\) (Singh, 1990) formation. In the current studies, these took 6-39 days to regenerate callus. Earlier the explants successfully regenerated in \(6-8 \ [S. \text{ platanifolium}\] (Singh, 1994)), \(8-10 \ [S. \text{ platanifolium}\] (Rama Rao, 1996)), \(14-20 \ [S. \text{ platanifolium}\] (Singh, 1990)), and \(15-20 \ [W. \text{ somnifera}\] (Rani and Grover, 1999)) days. During the present course of study, organogenesis was initiated in 11-45 days. Earlier, organogenic shoot bud initiation was reported in \(35-45 \ [S. \text{ platanifolium}\] (Singh, 1994)) and \(40 \ [S. \text{ platanifolium}\] (Ripudaman, 1996)) days.

The hypocotyl explants were placed in polar orientation, identical to that in the parent plant in the current study. The explants were placed horizontally on the medium surface and the orientation of the segments was identical to that of the whole seedling. Fári and Czákó (1981) kept the orientation of the hypocotyl segment explants of \(C. \text{ annuum}\) in a similar manner. Hypocotyl segments of \(S. \text{ platanifolium}\) were similarly placed horizontally on the medium (Singh, 1990). Bhatia \& Czákó (2004) stressed the need for medium sized explants with right orientation for regeneration purposes in \(Lycopersicon esculentum\).

During the course of the current study, the hypocotyl segments profitably regenerated on MS medium with and without growth regulators (4.4 \(\mu\text{M}\) each). A combination of BAP and IBA proved beneficial for initiation of regeneration, KN and BAP along with IAA for early response, and BAP in combined use with IBA for multiplication of somatic embryo formation and early shoot formation. Previously, the growth regulators proved optimal for regeneration in \(Capsicum\) (Gunay and Rao (1978), BAP and IAA), \(C. \text{ annuum}\) (Fári and Czákó (1981), BAP (2.0 mg l\(^{-1}\)) and IAA (1.0 mg l\(^{-1}\)) for callus regeneration; Valera-Montero and Ochoa-Alejo (1992), BAP (5.0 mg l\(^{-1}\)) and IAA (0.3 mg l\(^{-1}\)) for callus regeneration), \(S. \text{ laciniatum}\) (Bhatnagar \& Czákó (2004), KN (0.5 mg l\(^{-1}\)) and NAA (2.0 mg l\(^{-1}\)) for callus initiation and 0.5 and 1.0 mg l\(^{-1}\)), respectively for
callus proliferation and maintenance; BAP (4.0 mg l\(^{-1}\)) and IBA (0.25 mg l\(^{-1}\)) for shoot bud initiation], \textit{S. melongena} [Akhond and Bhuiyan (2001), 2,4-D for somatic embryogenesis (0.5 mg l\(^{-1}\)); Kamat and Rao (1978), NAA for callus initiation], \textit{S. platanifolium} [Singh (1990), BAP for both callus (1.0 ppm) and shoot bud (2.0 ppm) initiation, Rama Rao (1996) and Singh (1990), BAP (2.0 ppm) and NAA (1.0 ppm) for callus initiation], \textit{Withania somnifera} [Kulkarni \textit{et al.} (2000), BAP (0.5 mg l\(^{-1}\)) for multiple shoot initiation; Rani and Grover (1999) and Rani \textit{et al.} (2003), KN (0.2 mg l\(^{-1}\)) and 2,4-D (2.0 mg l\(^{-1}\)) for callus initiation].

In the present taxon, the callus was generally cream or green coloured, eventually becoming light brown to dark brown in colour. Green coloured callus was compact and subsequently underwent organogenesis, while the friable ones (formed in 2,4-D containing medium) did not lead to organogenesis. Singh (1990) reported the callus from \textit{S. platanifolium} segments to be greenish-white changing to light brownish-white with some hair-like roots. Callus obtained in \textit{S. platanifolium} was compact, hard and nodular in nature and changed from greenish-white to brownish-green with age (Singh, 1994). However, Rama Rao (1996) reported the change of hypocotyl callus colour from whitish-green to greenish with age in the above species.

The size and morphology of the hypocotyl explants changed after culturing in the present study; they remained green in colour but became slightly swollen or hypertrophied, especially at the excised ends making them look like dumb-bells. Regions of tissue hypertrophication were frequent sites of callus formation. Similar hypertrophy of the explants of \textit{Lycopersicon esculentum} was also observed by Zankowski and Rost (1990). They observed the hypertrophy at the excision site, near explant margins, and near vascular tissue. In the present study, the calli started from the cut ends subsequently encasing the explant. Likewise, the hypocotyl explants of \textit{L. esculentum} showed the formation of a slight barbell shaped callus. The calli of \textit{L. esculentum} were also reported to envelop the hypocotyl explant by Zankowski and Rost (1990). In the current study, the hypocotyls were given an excision at both the apical and basal ends and they responded from both the ends. Correspondingly, epicotyl and hypocotyl explants of \textit{L. esculentum} formed callus from the apical and basal ends (Zankowski and Rost, 1990).
5.1.1.2.2 Cotyledonary Leaf

In the present studies, the regeneration potential of cotyledonary leaves (along with the meristematic tip) procured from in vitro seedlings (15 day old) was assessed on full strength MS medium in the presence as well as absence of growth adjuncts. Previous workers have also tested the regeneration of these explants in Capsicum (Gunay and Rao, 1978), C. annuum (Manoharan et al., 1998), Lycopersicon esculentum (Velcheva et al., 2005), Nicotiana tabacum (Howard et al., 1983), Solanum melongena (Akhond and Bhuiyan, 2001; Saito and Nishimura, 1994), S. plataniolium (Rama Rao, 1996), and Withania somnifera (Rani et al., 2003).

The explants regenerated via both callus and shoot bud formation in the present studies. Similar observations were made earlier in Capsicum (Gunay and Rao, 1978). The explants generated either via callus formation in S. melongena ((Akhond and Bhuiyan, 2001; Saito and Nishimura, 1994) and S. plataniolium (Rama Rao et al., 1996) or shoot bud formation in C. annuum (Manoharan et al., 1998), L. esculentum (Velcheva et al., 2005) and N. tabacum (Howard et al., 1983).

In the present study, full strength MS medium was productively utilized for regeneration studies. MS medium was also used by Akhond and Bhuiyan (2001), Gunay and Rao (1978), Rama Rao (1996), Rani et al. (2003) and Saito and Nishimura (1994). However, Manoharan et al. (1998) successfully used half strength MS medium for their regeneration studies in C. annuum. Presently, growth regulators were not obligatory for callus and shoot bud regeneration. In majority of the earlier studies, the growth regulators proved obligatory for regeneration of these explants (Gunay and Rao, 1978; Rama Rao, 1996; Rani et al., 2003; Velcheva et al., 2005) whereas the regenerated callus both with and without auxin in S. melongena (Akhond and Bhuiyan, 2001).

Presently, the auxins IAA, NAA and 2,4-D (4.4 μM each) induced regeneration. The use of 2,4-D for inducing regeneration has been reported in Capsicum (Gunay and Rao, 1978) and S. melongena [Akhond and Bhuiyan, 2001; Saito and Nishimura, 1994 (50 μM)]. In the present study, shoot bud formation was supported by all the growth regulator combinations as well as their absence; BAP and IAA in combined use supported maximum number of multiple shoot formation. Shoot bud was supported in C. annuum (Manoharan et al., 1998, IAA along with TDZ), L. esculentum (Velcheva et al.,
Discussion

2005, zeatin along with IAA) and *N. tabacum* (Howard et al., 1983, combined use of KN and IAA). The use of cytokinins (BAP/KN) also supported regeneration; a good crop of shoots (an average of 20±20.41 and 24.25±16.86 shoots per explant in KN and BAP containing medium, respectively) was obtained in the current study. When KN was used in combination with auxins (IBA, IAA, NAA, and 2,4-D), their combined use proved synergistic. Multiple shoot formation was observed in combination containing IBA/IAA. Literature studies reveal the use of KN with 2,4-D [*W. somnifera*, Rani et al., 2003 (KN, 0.9 μM; 2,4-D, 9.1 μM)] for callus initiation and with IAA [*N. tabacum*, Howard et al., 1983 (IAA, 3.0 mg l⁻¹; KN, 0.3 mg l⁻¹)] for shoot bud initiation. Presently, BAP along with IAA proved beneficial for initiating an early regeneration response and for multiple shoot formation. Gunay and Rao (1978), similarly, used BAP along with IAA whereas Velcheva *et al.* (2005) utilized zeatin (1.0 mg l⁻¹) in the presence of IAA (0.1 mg l⁻¹) for inducing regeneration via shoot bud. Rama Rao (1996) utilized BAP and NAA (1.0 mg l⁻¹ each) for callus initiation. Manoharan *et al.* (1998), however, used TDZ (0.5 mg l⁻¹) in combination with IAA (0.5 mg l⁻¹) for inducing shoot bud regeneration in *C. annuum* cotyledon explants.

Currently, though medium containing KN and 2,4-D (4.4 μM each) induced cent per cent callusing in the explants, the calli failed to undergo organogenesis. Similarly, though maximum callusing was obtained in *Withania somnifera* cotyledon explants in medium supplemented with KN (0.9 μM) and 2,4-D (9.1 μM); the calli remained recalcitrant to regenerate regardless of the quality and combination of plant growth regulators used in the nutrient pool (Rani *et al.*, 2003).

In the present study, callus and shoot bud initiation was observed as early as 6.0 and 5.67 days, respectively. Similar observation of response by 7.0 days was reported by Rama Rao (1996) in *Solanum platanifolium*. In the current study, cotyledon explants responded with callus formation from the excision site at the base. Zankowski and Rost (1990) also showed that *L. esculentum* cotyledons reacted from near the cut site and veins.

5.1.1.2.3 Root

During the current studies, the regeneration of root tip explants procured from *in vitro* raised 15 d old seedlings positively responded to regeneration on full strength MS
medium with and without the growth additives. A perusal of literature reveals that only a few attempts have been made to evaluate the regeneration potential of these explants [Lycopersicon esculentum (Peres et al., 2001), Schizanthus hookerii (Jordan et al., 2006), Solanum melongena (Franklin et al., 2004), and Withania somnifera (Rani and Grover, 1999; Rani et al., 2003)]. MS full strength was fruitfully used during the current studies in consonance with the earlier works of Franklin et al. (2004), Jordan et al. (1996), Peres et al. (2001), Rani and Grover (1999), and Rani et al. (2003).

In the studies at hand, regeneration was via both callus and shoot bud formation in the medium devoid of any growth adjuncts; while their presence induced regeneration only via callusing. Peres et al. (2001) showed both the growth patterns in L. esculentum. Callus regeneration was obtained in Solanum melongena (Franklin et al., 2004) and W. somnifera (Rani and Grover, 1999; Rani et al., 2003) whereas shoot bud pathway was followed in Schizanthus hookerii (Jordan et al., 2006).

In the present studies, the combined use of BAP and NAA induced regeneration via callusing, though callus was non-organogenic. However, Jordan et al. (2006) reported multiple shoot bud regeneration in explants of Schizanthus hookerii in the above combination of growth regulators (2.69 μM NAA and 2.22 μM BAP). Presently, the combination of 2,4-D and KN lead to non-organogenic calli. Similar observations were obtained in W. somnifera [Rani and Grover, 1999; Rani et al., 2003 (9.1 μM 2,4-D and 0.9 μM KN)]. During the course of studies at hand, when BAP was used alone or in combination with IAA, organogenic calli was obtained. Likewise, organogenic calli was obtained on BAP (13.3 μM) and TDZ (0.45 μM) containing medium in Solanum melongena (Franklin et al., 2004); however, they also reported healthy shoot formation which were not obtained during the current studies.

The present studies were carried out under a photoperiod of 16 h L/D. Rani et al. (2003) reported callusing of W. somnifera explants both in light and dark conditions. In the current studies, it took 6-10 days for callus initiation and organogenic calli were obtained in 11-28 days; shoots were formed in 12-36 days of inoculation. Similarly, Franklin et al. (2004) reported the formation of organogenic calli and observed subsequent differentiation into shoot buds within 28 days in S. melongena.
The root explants hypertrophied at the excision sites; though their colour remained cream, changing in some cases to brown. The nutrient media, in which the root explant colour changed to brown, were not able to support a good callus response. During the first few days of culture, no sign of callus formation was visible, but within a week the explants slightly became thicker and formed callus. Similar hypertrophy at excision site and thickening of root explants was reported in *Lycopersicon esculentum* by Zankowski and Rost (1990).

5.1.1.2.4 Leaf


Full strength MS medium was utilized during the course of the present study which was similar to the earlier studies by Baburaj and Thamizhchelvan (1991), Bhatnagar et al. (2004), Okršlar et al. (2002), and Rani and Grover (1999). In the present context, regeneration pathway followed by the explants was either callus or shoot bud mediated or both. Callus mediated regeneration was observed in *C. annuum* (Kintzios et al., 1996), *N. tabacum* (Saha and Gupta, 1989), *S. platanifolium* (Rama Rao, 1996; Singh, 1994), *S. tuberosum* (Jayasree et al., 2001), and *W. somnifera* (Rani and Grover, 1999); whereas shoot bud regeneration pathway was followed in *L. esculentum* (Rao et al., 2005), *S. lacinatum* (Okršlar et al., 2002), *S. tuberosum* (Webb et al., 1983), and *W. somnifera* (Kulkarni et al., 1996). Both the patterns were followed in *S. lacinatum* (Bhatnagar et al., 2004), *S. tuberosum* (Gustafon et al., 2006) and *S. xanthocarpum* (Baburaj and Thamizhchelvan, 1991).

In the current study, NAA (2.2 μM) was fruitfully used for healthy callus formation and subsequent shoot formation. Similarly, callus formation and their
Callus formation was observed in NAA containing medium in *N. tabacum* (Saha and Gupta, 1989). Callus formation was observed in medium containing NAA in combination with BAP [*S. platanifolium* (Rama Rao, 1996; Singh, 1994), *S. tuberosum* (Jayasree et al., 2001), *S. xanthocarpum* (Baburaj and Thamizhchelvan, 1991) (0.5 mg l⁻¹ NAA and 2.0 mg l⁻¹)], KN [*S. laciniatum* (Bhatnagar et al., 2004) (2.0 mg l⁻¹ NAA and 0.5 mg l⁻¹ KN)]. In consonance with the present studies, NAA was found to be more effective for callus formation in *S. tuberosum* (Gustafon et al., 2006). NAA in combined use with trans-zeatin supported shoot bud formation in *S. tuberosum* (Gustafon et al., 2006), with BAP in *S. laciniatum* (Okrslar et al., 2002); with BAP alone supporting further shoot multiplication. In the present study, 2,4-D led to the formation of friable, sticky callus. Callus formation was reported in 2,4-D containing medium along with KN [*W. somnifera* (Rani and Grover, 1999) (2.0 mg l⁻¹ 2,4-D and 0.2 mg l⁻¹ KN)], BAP [*C. annuum* (Kintzios et al., 1996) (2.6-25.8 μM BAP and 2.5-9.8 μM IBA)]. During the course of the current study, it was observed that 2,4-D enriched medium formed friable callus while that containing NAA formed compact chlorophyllous callus. Jayasree et al. (2001), on a similar note, reported the formation of nodular callus in medium containing 2,4-D along with BAP and compact callus in the combined use of NAA and BAP in *S. tuberosum*.

Shoot buds were regenerated in the present study in IBA, NAA and IAA containing medium. IBA (2.2 and 4.4 μM) supported the formation of organogenic calli. Shoot bud regeneration was observed in IBA supplemented medium in combination with BAP [Bhatnagar et al., 2004 (4.0 mg l⁻¹ and 0.25 mg l⁻¹ IBA)]; NAA enriched medium in combined use of BAP [Okrslar et al., 2002 (10 μM BAP and 1 μM NAA)]; IAA along with BAP [Kulkarni et al., 1996]; GA₃ along with BAP [Baburaj and Thamizhchelvan, 1991 (4.0 mg l⁻¹ BAP and 2.0 mg l⁻¹ GA₃)]; NAA in combination with trans-zeatin [Gustafon et al., 2006 (1.0 mg l⁻¹ each)]. Bhatnagar et al. (2004) utilized 4.0 mg l⁻¹ concentration of BAP for shoot multiplication whereas Kulkarni et al. (1996) used 0.044 μM for the purpose.

The response was observed within 2 weeks of leaf culture in all the cases except the ones inoculated in IAA containing MS medium which responded a week later. Similarly, it took 10-15 days for callus initiation in leaf cultures of *S. platanifolium* (Rama Rao, 1996). Callus was visible after 10–14 days incubation, whereas shoots
Discussion


It was shown that the type of sugar supplied in the medium had a distinct effect on shoot organogenesis from cultured leaf explants of *S. melongena* by Mukherjee *et al.* (1991). Glucose or fructose at 44.0 mM was optimal in inducing shoot regeneration compared to sucrose. Sucrose at 11.0 and 22.0 mM induced more shoot organogenesis than at lower or higher levels. An additional 22.0 mM mannitol with 22.0 mM sucrose enhanced shoot regeneration significantly more than 22.0 mM sucrose alone. It is known that mannitol does not support tissue growth and is not metabolized, although it does enter callus tissue. The fact that sucrose level reaches an optimum at about half the optimal level of glucose or fructose could be because that the energy equivalent of 11.0 mM and 22.0 mM sucrose are 22.0 mM and 44.0 mM of the monosaccharides. 44.0 mM glucose yielded a significantly higher average number of shoots per explant than 22.0 mM sucrose which could be due to the fact that if glucose is the form of sugar immediately required for inducing organogenesis, supply of glucose could eliminate the energy required to breakdown sucrose into monosaccharide and 22.0 mM would have only half of the osmotic effect in the medium compared to 44.0 mM glucose. The sugar in the medium has a limited osmotic role to play in shoot organogenesis apart from being an energy source.

5.1.1.2.5 Shoot

In the current taxon, shoots (2-3 cm long) procured from *in vitro* raised plantlets were used as explants for regeneration studies. A perusal of previous studies reveals that shoot explants were successfully utilized earlier for the regeneration of *in vivo* sourced *Atropa belladonna* (Jaziri *et al.*, 1994; Kamada *et al.*, 1986) and *in vitro* sourced *Datura innoxia* (Zayed *et al.*, 2006), *Lycium chinensis* (Liu, 1991), *Lycopersicon esculentum* (Pugliesi *et al.*, 1999), *Nicotiana tabacum* (Locatelli *et al.*, 2003), *Scopolia parviflora* (Kang *et al.*, 2004), *Solanum acaule*, *S. commersonni*, *S. tuberosum* (Anjum and Villiers, 1991).
Discussion


During the studies at hand, full strength MS medium was made use of for regeneration in shoot explants. Similarly, Anjum and Villiers (1997), Liu (1991), Rama Rao (1996), Rani and Grover (1999), Westcott et al. (1977), and Zayed et al. (2006) utilized full strength MS medium for their studies. However, Jaziri et al. (1994) advocated the use of half strength MS medium for regeneration in *in vivo* shoot explants of *A. belladonna*. Kang et al. (2004) used B5 medium for regeneration studies in *Scopolia parviflora*. Sucrose was fruitfully added at 3% concentration in the present studies. However, 8% sucrose was utilized by Anjum and Villiers (1997) in *Solanum acaule*, *S. commersonii*, and *S. tuberosum*; whereas Dobigny et al. (1996) used 5.8% sucrose for regeneration of *S. tuberosum* explants.

Currently, callus as well as shoot bud mediated regeneration patterns was followed. Earlier, only callus mediated regeneration was reported in *D. innoxia* (Zayed et al., 2004), *Lycium chinensis* (Liu, 1991), and *S. platanifolium* (Rama Rao, 1996) while regeneration occurred only via shoot bud formation in *Scopolia parviflora* (Kang et al., 2004), *Solanum acaule*, *S. commersonii*, and *S. tuberosum* (Anjum and Villiers, 1997), and *S. tuberosum* (Westcott et al., 1977). Rani and Grover (1999) reported regeneration via both the patterns in *W. somnifera*.

In the present study, plant growth regulators were used for callus and shoot bud formation; 2,4-D (4.4 μM) was unable to induce any shoot bud formation and responded with only callus and root formation. The combined use of 2,4-D with KN [*W. somnifera* (Rani and Grover, 1999) (2.0 mg l⁻¹ 2,4-D and 0.2 mg l⁻¹ KN)] and NAA [*Lycium chinensis* (Liu, 1991) (0.2 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ NAA)] showed callus formation while its combined use with BAP was successful in producing microtubers in *S. acaule*, *S. commersonii* and *S. tuberosum* (Anjum and Villiers, 1997). During the study at hand, BAP (4.4 μM) was most fruitfully utilized in inducing multiple shoots. Likewise, BAP (22.19 μM) supported speeding up of microtuber production in *S. tuberosum* (Anjum and Villiers, 1997). They, however, advocated the combined use of BAP (22.19 μM) and 2,4-D (2.26 μM). Rani and Grover (1999) also used BAP (2.0 mg l⁻¹) to form shoot buds from shoot explant callus of *W. somnifera*. In the present study, IAA (4.4 μM) formed
callus and some shoot buds. BAP (1.0 mg l\(^{-1}\)) in combined use with IAA (0.5 mg l\(^{-1}\)) exhibited callus formation from Datura innoxia explants which differentiated in growth regulator-free medium (Zayed et al., 2006).

Presently, shoot cultures were customarily and profitably maintained under a 16 h photoperiod under white light. However, Bhatia et al. (2004) studied the effect of incubation of shoot cultures of Lycopersicon esculentum under different qualities of light and concluded that white light showed better results than red or green light and a 16 h photoperiod was optimum. Similarly, Rama Rao (1996) reported successful use of 12 h photoperiod in S. platanifolium. The use of light was recommended by Pugliesi et al. (1999) in L. esculentum. Dobigny et al. (1996) and Locatelli et al. (2003) also advocated the use of 16 h photoperiod in shoot cultures of S. tuberosum and N. tabacum, respectively. Jaziri et al. (1994) and Kamada et al. (1986) similarly used 16 and 18 h photoperiod during in vivo shoot explants of Atropa belladonna.

The first visible signs of callus or shoot bud formation were evident on the explants after one week of culture during the current investigations. However, callus initiation occurred after 12-15 days and 6 weeks in S. platanifolium (Rama Rao, 1996) and 6 weeks in D. innoxia (Zayed et al., 2006) stem explants.

5.1.1.2.6 Multiple shoot regeneration

Shoot explants were productively used for raising multiple shoots in the present study. Since the shoot explants gave the best response in BAP and KN containing medium, further experiments were designed at different concentrations of the two growth regulators (2.2, 4.4 and 8.8 \(\mu\)M). Similarly, axillary shoots were used by Sanatombi and Sharma (2007) (Capsicum frutescens) and Saritha and Naidu (2007) (Withania somnifera). Nonetheless, various other explants like nodes and internodes (W. somnifera; Kulkarni et al., 2000), seedling explants (C. annuum; Kumar et al., 2005 b), leaf [Solanum aculeatissimum, S. aviculare, S. gile, S. khasianum, S. sisymbriifolium, and S. torvum (Gleddie et al., 1985)], and callus from axillary shoot base (W. somnifera; Rani and Grover, 1999) were utilized for the purpose.
In the current study, full strength MS medium was utilized for multiple shoot regeneration in consonance with earlier studies (Gleddie et al., 1985; Kulkarni et al., 2000; Kumar et al., 2005 b; Rani and Grover, 1999; Sanatombi and Sharma, 2007).

Presently, medium containing BAP and KN were utilized separately for multiple shoot generation and 4.4 and 8.8 µM concentration was considered to be optimal in each case. BAP, singly [C. frutescens, Sanatombi and Sharma (2007) (8.8-44.4 µM); W. somnifera, Kulkarni et al. (2000) (0.5-1.0 mg l⁻¹); Rani and Grover (1999) (2.0 mg l⁻¹)] or in combination with IAA [C. annuum, Kumar et al. (2005 b) (26.63 µM BAP and 2.28 µM IAA); C. frutescens (Sanatombi and Sharma (2007) (8.8-44.4 µM BAP and 5.7, 28.5 µM IAA)], NAA [W. somnifera, Saritha and Naidu (2007) (2.0 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA)], TDZ [W. somnifera, Kulkarni et al. (2000) (0.2-0.3 mg l⁻¹)], KN [C. frutescens, Sanatombi and Sharma (2007) (22.2 µM BAP and 4.65 µM KN)] was also used. KN alone (9.3-46.7 µM) was also utilized for the purpose by Sanatombi and Sharma (2007) in C. frutescens.

An average number of 54.75±8.92 and 48.5±6.29 shoots per explant were obtained in BAP and KN (8.8 µM each) enriched medium, respectively in the current study. Kumar et al. (2005 b) reported 20-25 shoots per explant in C. annuum whereas 5.6 shoots were recorded in C. frutescens (Sanatombi and Sharma, 2007). Initiation of shoot buds was observed in 1.75 to 2.27 weeks in the study at hand. Likewise, Sanatombi and Sharma (2007) reported shoot initiation in 2 weeks in C. frutescens.

The capacity of shoot bud differentiation and shoot proliferation varied with the quality and quantity of the growth regulators (KN/BAP); requiring a higher concentration of cytokinins. In the same way, Varisamohamed et al. (1998) reported the dependence of shoot formation on variation in the plant growth regulators.

In the present scenario, it was noticed that out of the auxins used, 2, 4-D was the least useful in terms of callus production or organogenesis and regeneration from leaf as well as root explants. The callus formed was sticky and cream and was able to grow at a very slow rate. Even in the hypocotyl explants, it produced only a cream callus without any regenerative competence. Similarly, Gunay and Rao (1978) reported the regeneration of only a friable callus in MS medium fortified with 2,4-D in Capsicum. In contrast, 2,4-D has been shown to be effective singly or in combination with other growth regulators.
Discussion

for regeneration purposes by various authors [Akhond and Bhuiyan (2001); Anjum and Villiers (1997); Liu (1991); Rani and Grover (1999); Rani et al. (2003); and Saito and Nishimura (1994)].

It is evident from the above results that the texture of calli depended not only on the nature of explant but also on the culture medium constituents. Friable and compact nodular calli were induced from different explants. Similar views were also expressed by Rani and Grover (1999); Rani et al. (2003); and Saito and Nishimura (1994).

Our results have indicated that the nature and concentration of auxin have a pronounced effect on callusing of the explant. The influence of growth regulators and other organic supplements was also shown in other members of Solanaceae by various workers such as Akhond and Bhuiyan (2001); Kamada et al. (1986); Kang et al. (2004); Kintzios et al. (2000); Rama Rao (1996); Rani and Grover (1999); Rani et al. (2003); Saito and Nishimura (1994); Singh (1994). The concentration of growth regulators used varies with the species and cultivar and the specific regulator made use of. There have also been various reports on the combined utilization of auxins and cytokinins in solanaceous species. Kamat and Rao (1978) stated that cytokinin - auxin interaction either promoted or inhibited the development of shoots and roots and this depended upon the ratio of the hormones in the medium. Induction of organ formation in tissue cultures is a complex process brought about by the interaction of a number of factors. The differentiation of shoots and roots or unorganised proliferation from unorganised callus in S. xanthocarpum was studied by Rao and Narayanaswami (1968). It was found that when callus habituated to 2, 4-D and meso-inositol were transferred to an auxin-free medium or to a medium where auxin was reduced and meso-inositol was increased, root and shoot differentiation was promoted. Some tissues tend to lose their ability to initiate organs on repeated sub-culture and this phenomenon is due to the loss of a specific factor contained in the primitive explant which is no longer synthesised in vitro. Here an incorporation of a balanced ratio of supplements or the deletion of one or the other in the basal medium could bring about cellular differentiation leading to organ formation in callus tissues which were under continued subculture and had apparently lost their morphogenetic potentialities.
In the present set of investigations, calli and organogenic calli or multiple shoots were raised from a variety of explants, namely; hypocotyls, cotyledonary leaves, and root-tips from the \textit{in vitro} seedlings; shoot and leaf explants from adult \textit{in vitro} raised plantlets. This diversity in the tissue explants from which regeneration was attained led to an enhanced flexibility in regeneration protocols for the present species. The type of explant, their physiological age as well the growth regulator constituents of the medium influenced the rate and quantity of callus formation in the present study. In the present scenario, hypocotyl and leaf explants were the best for callus formation and organogenesis. However, root explants were slower to respond and also the volume of callus formed was lesser. From the adult explants, shoot and leaf explants were quick to respond with a good volume of callus and multiple shoot formation. In consonance with the present investigations, various authors have stated that the callus formation was affected by the explant type and plant age. Ercan \textit{et al.} (2006) investigated the effect of growing season and donor plant age on anther culture in \textit{Capsicum}. Similarly, Kintzios \textit{et al.} (2000) also studied the effect of the explant position on the donor plant on the induction, proliferation and development of somatic embryos from young, fully expanded leaves of \textit{C. annuum}. An interaction has been found between genotype, explant type, and the age of explant donor plant for shoot regeneration rate and the number of shoots produced per explant. Regeneration response is also influenced by the time of the year and the environment where the source plant was grown. The response of tomato explants from seedlings grown in \textit{in vitro} culture differed from those raised in a greenhouse (cf. Bhatia \textit{et al.}, 2004). The type of explants used not only determines the proportion of explants which show organogenesis, but also the number of shoots produced per explant. Plastira and Perdikaris (1997) reported differential regeneration frequency of various explants in the order of hypocotyls, cotyledons, and leaves (in descending order). Preferential regeneration was also determined from hypocotyl explants better than from cotyledon explants (Gunay and Rao, 1980). Contrasting views were expressed by Schutze and Wieczorrek (1987) who attributed cotyledon explants with better regeneration capacity than hypocotyls. Similar to the present opinion, Kantharajah and Golegaonkar (2004) stated that different factors such as genotype, explant type and position, growth regulators, polyamines and some other factors affect somatic embryogenesis. The
Discussion

regeneration and proliferation potential of the different explants varied with respect to the explant as well as the growth regulator provided in the nutrient medium during the present set of investigations. The proliferative capacity of cells of the explant might be governed by several factors including endogenous concentration of growth regulator at the time of excision, capacity to synthesise growth regulators and essential metabolites, and sensitivity to exogenous growth regulators (cf. Zankowski and Rost, 1990). Rani and Grover (1999) also demonstrated the variability in the regeneration potential of the different explants of *W. somnifera*, with axillary shoots being the best and root segments being the worst. Growth as well as differentiation was found to be better in case of leaf calli than in calli obtained from hypocotyl and stem explants of *S. platanifolium* (Singh, 1994).

In choosing a medium to promote callus growth, three criteria needed to be met to facilitate the maintenance and multiplication of cultures; reduced shoot formation, prolific callus growth, and the ability to regenerate shoots when removed from the callusing medium. Shoot development in *Prunus avium* callus was affected by the choice of basal medium (Feeney et al., 2007). By using the *in vivo* shoot tips of *P. avium* as explants, Feeney et al. (2007) avoided the lengthy time and resources required for initiation, establishment, and maintenance of large stocks of shoot cultures for providing tissues. However, in the present study no *in vivo* sourced explants were utilized for the regeneration purposes. The explants were taken only from the *in vitro* sources during the course of investigation. The latter approach was advantageous as the tissues were available throughout the year/the course of the study, shoots were maintained in a controlled/supervised environment, and it did not lead to any losses because of contamination and higher phenolics from the field-grown explants. Sterilising the field-grown tissues and transferring them to the tissue culture environment also imposes a stress on the explants, which might disrupt or inhibit and delay the response from the *in vivo* explants.

5.1.1.3 Rooting response

5.1.1.3.1 Effect of growth regulators

In the present study, shoots (2.0-3.0 cm long), procured from *in vitro* shoot culture or plantlets, and were used for initiating rooting. Similarly, root formation was

In the studies at hand, rooting response was initiated in full strength MS medium with and without growth regulators. Likewise, root formation was initiated in growth regulator-free MS medium in *C. annuum* and *Solanum laciniatum* (Okršlar *et al.*, 2002). However, half strength MS medium was utilized for rooting in *W. somnifera* (Kulkarnia *et al.*, 2000) and *C. annuum* (Manoharan *et al.*, 1998); half strength B5 medium for *Scopolia parviflora* (Kang *et al.*, 2004). Shimomura *et al.* (1991) used liquid MS medium without regulators in *H. albus* and *H. niger*. However, sand along with half strength MS medium was made use of in *C. annuum* (Valero-Montero and Ochoa-Alejo, 1992) while Franklin *et al.* (2004) utilized soilrite and water in *S. melongena* for the purpose.

Currently, IAA at 8.8 μM concentration was optimal for an early rooting response as well as an exceptionally high number of roots per explant. IAA was used alone in *C. annuum* [Manoharan *et al.*, 1998 (0.5 mg l⁻¹)]; *S. xanthocarpum* [Baburaj and Thamizhchelvan (1991) (4.0 mg l⁻¹)], or in combination with IBA [*W. somnifera* (Rani and Grover, 1999) (2.0 mg l⁻¹ each)]. IAA or IBA was utilized for the purpose in *C. frutescens* [Sanatombi and Sharma, 2007 (2.8 μM IAA or 2.4-4.9 μM IBA)]; while IAA or NAA was made use of in *C. annuum* (Gunay and Rao, 1978) and *D. innoxia* (Shimomura *et al.*, 1991). During the present study, NAA did not have root promoting effect (except at lower concentration of 2.2 μM). However, the plant growth regulator was successfully used alone by Kamat and Rao (1978) in *S. melongena*. In the current study, IBA (8.8 μM) was beneficial in eliciting a rooting response with longer roots while the lower concentrations of 2.2 and 4.4 μM also responded with rooting. Similarly, the
growth regulator was used for root initiation in *N. tabacum* [Saha and Gupta (1989) (2.0 mg l$^{-1}$)], *W. somnifera* [Rani and Grover (1999) (2.0 mg l$^{-1}$); Rani *et al.* (2003) (2.0 mg l$^{-1}$/9.9 µM)]. IBA was also used in combination with NAA [*Symonanthus bancroftii* (Panaia *et al.*, 2000) (1.0 mg l$^{-1}$ each)], IAA [*W. somnifera* (Rani and Grover, 1999) (2.0 mg l$^{-1}$ each); (Wadegaonkar *et al.*, 2006) (9.85 µM IBA and 2.85 µM IAA)]. Cytokinins were not utilized for the purpose of root initiation during the course of the present study, though there are some reports on their successful utilization. BAP was used for rooting purposes in *W. somnifera* [Kulkarni *et al.* (1996) (0.044 µM)]. Rooting was observed in 72–94% of shoots obtained from TDZ-containing regeneration medium (Peddaboina *et al.*, 2006).

During the course of present study, it was found that the higher concentration of IBA (4.4 and 8.8 µM) gave more rooting response while a different pattern was observed regarding the number of roots per explant and root length where 8.8 µM was the best followed by lower concentration of 2.2 µM and 4.4 µM, respectively. However, in the case of IAA, all the three concentrations gave the same per cent of rooting response while highest concentration (8.8 µM) responded with a remarkably high number of roots per explants; the pattern followed in root length was 8.8, 2.2 and 4.4 µM in ascending order. Panaia *et al.* (2000) reported 31% rooting in *Symonanthus bancroftii* in medium containing 0.5 µM each of IBA and NAA while 36% when the concentrations were raised to 1.0 µM. It has been found that action of auxin in root is similar to that of stem but the concentrations of auxin stimulatory to stem growth are inhibitory to root growth. Roots are sensitive to auxin than stem and low concentration can bring root elongation. The application of relatively high concentration of IAA to roots, not only retard root’s elongation but cause a remarkable increase in number of branches of roots. This has led to the commercial application of IAA to promote root formation in cuttings of economic plants (Shukla, 1978).

In the study at hand, rooting response was initiated in 9.75-15 days. Similarly, Baburaj and Thamizhchelvan (1991) reported development of roots in 10-15 days in *Solanum xanthocarpum*. Direct rooting was observed in *W. somnifera* explants within 8-10 days (Wadegaonkar *et al.*, 2006). As many as 66.67% explants responded with root generation in the current study. Peddaboina *et al.* (2006) reported 72-94% rooting in *C.*
Discussion

annuum whereas it ranged from 31-36% in Symonanthus bancroftii (Panaia et al., 2000) and 87% in W. somnifera (Rani et al., 2003).

Maximum number of roots per explants observed was 26 (IAA at 8.8 μM concentration) followed by 10 roots per explant (IBA at 8.8 μM concentration). Wadegaonkar et al. (2006) reported an average 9.2 roots per explant in W. somnifera in medium containing IAA (2.85 μM) and IBA (9.85 μM).

5.1.1.3.2 Effect of IBA and AC on rooting

Presence of AC (0.2%) in basal MS medium as well as IBA fortified medium proved inhibitory for the initiation of rooting response with respect to root number per explant as well as root length in the current study. This was probably due to the partial absorption of auxin and other nutrients by activated charcoal. Activated charcoal is involved in a number of stimulatory and inhibitory activities including the release of substances naturally present in AC which promote growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene. The effect of AC on growth regulator uptake is still unclear but some workers believe that AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants. Thomas (2008) opined in a similar way. Similar studies on the effect of AC on rooting have not been reported in the members of Solanaceae. Activated charcoal in the culture medium improved axillary bud proliferation of Lavendula and growth of Anemone seedlings, but it negatively influenced root formation (Mensuali-Sodi et al., 1993). The beneficial influence of AC (20 g L⁻¹), added to the basal culture medium, was noted for in vitro growth and further rooting of microcuttings collected from juvenile clones of Sequoiadendron giganteum (Bon et al., 1988). The addition of activated charcoal to the water extracts from Agar Bacteriological and Agar Commercial Gel had no effect on their root-promoting ability in mungbean. Extracts with exogenous IBA which were treated by autoclaving or via a freeze–thaw cycle, significantly increased rooting. However, incorporation of activated charcoal to similar IBA-containing extracts reduced rooting (Arthur et al., 2006).
5.1.1.3.3 Effect of the size and age of shoot explants on rooting

The effect of size and age of shoot explants on their root forming capability was also assessed during the current study. IBA containing medium showed the maximum number of roots in ‘nt’ and ‘pts’ explants (9.44 and 8.46 roots per explant, respectively). In IBA containing medium, ‘vst’ explants exhibited the formation of longest roots (6.84 cm long) followed by ‘nt’ explants (6.65 cm long). Some explants also formed callus at the base of the cut end of the explants. It could be concluded, therefore, that the youngest ‘vst’ explants were not able to elicit a root differentiation response; while ‘nt’ explants even without a shoot tip formed the maximum number of roots closely being trailed behind by ‘pts’ explants. Addition of AC proved detrimental to the rooting response of the explants in the presence of IBA, proving its non-obligatory presence for root initiation. Its presence also led to a decrease in the root length irrespective of the explant type. In the present study, earliest root formation response was observed in 3.04-4.35 days and cent per cent explants responded with root formation. No study has been carried out to study the impact of the explant type, age and length and that of activated charcoal on the frequency of root formation and length of the roots in the members of Solanaceae.

5.1.1.4 Lab to Land transfer of in vitro plantlets

During the current study, in vitro plantlets with healthy root system were transferred to ex vitro conditions directly with success. In the same way, transfer to land conditions was carried out in Capsicum annum (Gunay and Rao, 1978; Peddaboina et al., 2006), C. frutescens (Sanatombi and Sharma, 2007), Scopolia parviflora (Kang et al., 2004), Solanum melongena (Franklin et al., 2004; Kamat and Rao, 1978), S. platanifolium (Singh, 1990), S. tuberosum (Kowalski et al., 2006; Westcott et al., 1977), Symonanthus bancroftii (Panaia et al., 2000), and Withania somnifera (Kulkarni et al., 1996). However, Rani and Grover (1999) initially transferred the plantlets of W. somnifera to soil mixture in tissue culture room followed by their transfer to glass-house. Currently, acclimatization procedure also led to positive results.

In the present study, the acclimatized plants productively grew in soil mixture containing peat moss, river sand, and silica in a ratio of 1:1:1; in addition to spraying with half MS medium (only the major salts and iron source) initially and later with distilled
Various workers made use of different soil mixtures; sand: clay: farm yard manure \[ \text{Solanum platanifolium} \text{ (Singh, 1990) (50:25:25)}, \] peat moss: vermiculite \[ \text{Scopolia parviflora} \text{ (Kang et al., 2004)}], sand: soil \[ \text{W somnifera} \text{ (Kulkami et al., 1996; Rani and Grover, 1999; Rani et al., 2003) (1:1)} \]. During the current study, river sand alone was also successfully utilized for acclimatization of the \text{in vitro} \text{ plantlets. Sand alone was used earlier for the intention by Gunay and Rao (1978) (C. annuum), Kamat and Rao (1978) (S. melongena), Peddaboina et al. (2006) (C. annuum), Sanatombi and Sharma (2007) (C. frutescens), and Westcott et al. (1977) (S. tuberosum). In the current study, half strength MS medium was sprayed on the transferred plantlets, however, Singh (1990) added Knop’s medium to the sterilized sand culture for the transfer of \text{Solanum platanifolium}. The \text{in vitro} \text{ shoots were subjected to MS medium in the presence or absence of growth regulators for the initiation of roots leading to complete plantlet formation before their lab to land transfer. However, shoots of S. melongena were rooted in water under \text{in vivo} \text{ or \text{in vitro} \text{ conditions (Franklin et al., 2004). Conde et al. (2008), however, advocated the use of the method where the \text{in vitro} \text{ shoots of Ulmus minor (Elm) (with thick stems and hard leaves) were directly shifted for acclimatization, and reported that there was no need for a previous \text{in vitro} \text{ rooting step and direct acclimatization could effectively reduce time and cost involved. In a similar way, \text{in vitro} \text{ shoots of C. annuum were excised and rooted on coarse sand, wetted with half strength MS medium (Valera-Montero and Ochoa-Alejo, 1992).}}}

During the present investigations, the acclimatized plantlets of \text{S. xanthocarpum} \text{ grew to maturity, flowered and formed berries. Seed formation in acclimatized plantlets has also been reported in C. annuum (Peddaboina et al., 2006), S. melongena (Franklin et al., 2004; Kamat and Rao, 1978), and S. tuberosum (Kowalski et al., 2006). The \text{ex vitro} \text{ survival rate of acclimatized plantlets was 92\% in the current study. Similarly, Kulkarni et al. (1996) and Panaia et al. (2000) reported up to 100 and 90\% survival in \text{W. somnifera} \text{ and \text{Symonanthus bancroftii}, respectively; while 50 and 60\% survival was recorded in \text{Solanum platanifolium} \text{ (Singh, 1990) and \text{W. somnifera} \text{ (Rani et al., 2003), respectively.}}}

}
5.1.2 *Malaxis acuminata*

5.1.2.1 Seed Germination

*In vitro* seed germination represents the most efficient method of orchid propagation for conservation purposes (Pathak *et al.*, 2001; Seeni and Latha, 2000; Stewart and Kane, 2006; Vij, 1995). In the present study, asymbiotic seed germination was carried out to assess the germination potential of immature as well as mature seeds (at different stages of development) in addition to testing the effect of various growth adjuncts at different concentrations. The ability of orchid embryos to germinate prior to reaching maturity, first demonstrated by Withner (1943), led to development of 'green-pod' culture technique adding new dimensions to orchid propagation. The utility of the technique lies not only in the ease of surface sterilization but also in better frequency of germination (Vij *et al.*, 1995).

In the study at hand, asymbiotic germination of the terrestrial orchid *Malaxis acuminata* seeds was successfully tested. Knudson (1922) first germinated orchid seeds asymbiotically. However, orchid seed germination studies are often viewed as unreliable or unrealistic since little is known concerning the germination and *in vitro* seedling developmental requirements of terrestrial orchids. Orchid species require eight to eleven years of *ex vitro* growth before reaching reproductive maturity. To overcome these problems, the development of optimized asymbiotic seed germination methods for entire orchid genera or individual species are suggested.

5.1.2.1.1 Effect of stages of seed development

Seeds at different stages of development (12, 16 and 20 wap) procured from green and brown unburst capsules (pods) were used productively for germination studies (Fig. 392). Seeds procured from green pods were successfully germinated earlier by various workers in *Aerides multiflorum* and *Coelogyne punctulata* (Katiyar *et al.*, 1987), *A. odorata* (Pant and Gurung, 2005), *Cephalanthera falcata* (Yamato and Iwase, 2008), *Coelogyne punctulata* and *Cymbidium elegans* (Sharma and Tandon, 1990), *Cymbidium lowianum* (Nongdam *et al.*, 2006), *Cymbidium* hybrids (Nagaraju *et al.*, 2002), *Cymbidium elegans*, *Coelogyne prolifera*, *C. cristata*, *C. porrecta*, *A. multiflorum*, *C. lowianum*. 

151
Sarcanthus pellidus, Bulbophyllum cosmosus, and Thunia alba (Sharma and Tandon, 1987), Dendrobium hybrid (Lekha Rani et al., 2005), Geodorum densiflorum (Sheelavantmath et al., 2000), Peristeria elata (Bejoy et al., 2004), and Vanda amesiana (Devi et al., 2006). In the present study, it was found that the seeds had a higher germination potential with increase in the germination potential with the maturity and the development of the seeds. The germination potential was found to increase with the age of the capsule with the most mature ones exhibiting the maximum germination percentage (70, 97, and 97% germination in seeds inoculated 12, 16 and 20 wap, respectively). In a similar way Muralidhar and Mehta (1985) and Singh et al. (2006 b) successfully used the seeds procured from un-dehisced but almost mature pods of Cymbidium longifolium and Aerides odorata, Dendrobium Dactylorhiza pierardii, Phaius tankervilliae, and Rhynchostylis retusa, respectively. Stewart and Kane (2006), similarly, successfully germinated the seeds of Habenaria macroceratitis prior to dehiscence of the mature capsules. Bhadra and Hossain (2004), Bhadra et al. (2005), Gayatri and Kavyashree (2007), and Talukdar (2001), likewise, used mature pods of Micropera pallida, V. tessallata, Epidendrum radicans, D. aphyllum. Mature seeds have been germinated in vitro successfully by earlier authors [Vej sadova, 2006 (Dactylorhiza incarnata subsp. serotina, D. maculata subsp. maculata, Liparis loeselii); Dutra et al., 2008 (Bletia purpurea)]. Shimura and Koda (2004) opined that immature seeds were not suitable for tissue culture experiments, because the seeds easily lost their germination capability within a few days after the fruit’s harvest while the mature seeds kept their capability at least for a few months. Usually mature seeds are in dormant state just after harvest and chilling the seeds for several weeks could break the dormancy (cf. Shimura and Koda, 2004). Similar to the present studies, Kitsaki et al. (2004) also investigated the effect of seed maturity in Ophrys species.

During the present studies, seeds procured 20 wap showed an overall maximum percentage of seed germination as well as seedling formation than the seeds procured at 12 and 16 wap. The immature embryos germinate better than the mature ones, however, very young ovules fail to germinate due to dormancy, pH, and other metabolic factors (Arditti, 1967). However, presently, seeds procured after 20 wap and mature seeds after the dehiscence of capsules could not be tested, due to the paucity of material and time. A
During the current investigations, it was observed that under dark conditions or when the nutrient medium was insufficient, rhizoidal hairs appeared on the protocorms and remained till the whole protocorm developed chlorophyll. Under natural conditions, seeds of terrestrial orchids are known to produce copious rhizoids (cf. Stewart and Kane, 2006). Rhizoids or ‘absorbing hair’ are used as entry points during infection by fungal mycorrhizae, and would be essential for the prolonged existence of protocorms in nature. Stewart and Kane (2006) found that protocorms of *Habenaria macroceratitis* growing in continuous darkness formed long rhizoids.

5.1.2.1.2 Nutrient medium

The most important aspect of seed/embryo culture is the selection of the right culture medium that would support progressive and orderly development of embryos. To satisfy the nutritional requirements of orchid embryos during germination and subsequent development into seedlings *in vitro*, a large number of culture media have been devised and many of these are species specific (Arditti et al., 1982). During the course of the present investigations, full strength M and MS media were fruitfully used for assessing the germination and morphogenetic potential of the seeds. Literature studies reveal that several media such as B₅, Hyponex, Kano, KC, Lindemann, modified Lucke and Malmgren, Malmgren modified terrestrial orchid and PhytoTechnology orchid seed sowing medium, M, MS, Nitsch, Pfeffer’s, Phytamax, and VW have been successfully utilized for orchid seed germination [Bejoy et al. (2004), Bhadra et al. (2005), Chang et al. (2004), Dutra et al. (2008, 2009), Gayatri and Kavyashree (2007), Hossain (2008), Kalimuthu et al. (2007), Katiyar et al. (1987), Kauth et al. (2006), Mahendran and Narmatha Bai (2009), Mathews and Rao (1985), Men et al. (2003), Montri et al. (2008), Muralidhar and Mehta (1985), Nagaraju et al. (2002), Nongdam et al. (2006), Pant and Gurung (2005), Seeni and Latha (2000), Sharma and Tandon (1987, 1990), Shimada et al. (2001), Shimura et al. (2007), Singh et al. (2006 b), Singh et al. (2006 b), Stewart and Kane (2006, 2007), Talukdar (2001), Xu et al. (2001), Yamazaki and Miyoshi (2006)]. However, half strength MS medium was utilized in *Bletia purpurea* and *Cyrtopodium punctatum* (Dutra et al., 2008, 2009), *Dendrobium* hybrid (Lekha Rani et al. (2005), *D. candidum* (Zhang et al., 1993), and *D. lituiflorum* (Chang et al., 2004). No media
Discussion

composition can be considered to be useful for all the orchid species and the nutrient demands are species specific (Mitra, 1967).

When the efficiency of M and MS media was assessed, basal MS medium was found to be the best and self-sufficient in inducing a high percentage of seed germination in the current study. However, the morphogenetic entities failed to develop roots in both M and MS media when used without growth adjuncts; though healthy protocorm and PLB multiplication was observed. Similar to the present studies, Pant and Gurung (2005) conclusively stated that the basal MS medium was better than the one containing plant growth regulators for the in vitro seed germination, higher number of protocorm formation and subsequent seedling development in Aerides odorata. However, Salwan (1992) reported better shoot growth in Acampe carinata, Aerides multiflorum, Phaius flavus, and Rhynchostylis retusa in M medium which has higher ratio of manganese and iron ions than that in that found in MS medium.

In the present study, seeds germinated in MS medium overall exhibited a higher percentage of seed germination than those growing in M medium though the difference was quite insignificant, the former having a higher concentration of both macro and micronutrients. Likewise, Bhadra et al. (2005) reported higher frequency of seed germination in Vanda tessellata in MS medium compared to Phytamax medium. The results of Sharma and Tandon (1987) were also in consonance with the present results with KC medium being better for seed germination as compared to Pfeffer’s and VW media; the former being quite rich in macro- and micro-nutrients and it also contained sucrose. Mahendran and Narmatha Bai (2009) also reported that MS medium was better than Knudson C and Knudson C modified Morel media for the germination of immature seeds of Satyrium nepalense.

5.1.2.1.3 Carbohydrate source

The significance of carbohydrates and other growth adjuncts is being realized in in vitro studies. The carbohydrates are well known source of energy and their exogenous supply is important as the young orchid seeds are incapable of converting the stored lipids into simpler carbohydrates. Many studies have been sustained on the carbohydrate nutrition on the immature and mature orchid embryos. While sugars have been regularly used as
source of carbohydrate (Arditti, 1967; Bahme, 1949; Wynd, 1933), employment of corn starch in the presence of tomato juice in the medium has also been shown in the embryos of *Cattleya, Dendrobium, Epidendrum*, and *Phalaenopsis* seeds (Ito, 1951). The significance of the type and concentration of sugar in promoting orchid seed germination and seedling growth has been estimated (Arditti *et al.*, 1972). The root and shoot ratio is significantly affected by sucrose concentration in several orchid embryos (Yates and Curtis, 1949). Many sugars other than glucose, namely fructose and sucrose are acceptable source of carbohydrates for orchid seed germination (Wynd, 1933). Warcup (1974) demonstrated that the orchid seeds fail to germinate in a mineral solution, unless subjected to a suitable carbohydrate source. Similarly, Sharma and Tandon (1990) also noticed very poor germination and seedling growth in *Coelogyne punctulata* and *Cymbidium elegans* in sugar free medium and emphasized the importance of sugars in promoting orchid germination and growth. According to Arditti and Ernst (1984), the larger size of a polysaccharide molecule poses permeability problems, an extra-cellular hydrolytic enzyme may be needed during germination. The monosaccharides, on the other hand, serve as a good source of carbohydrate for germinating orchid seeds. Sucrose was successfully used at 2% concentration as the sole carbohydrate source in the present study irrespective of the medium used. In accord with the present results, 2% sucrose was utilized by various workers [Bejoy *et al.*, 2004; (Peristeria elata), Gupta, 2003; (Cymbidium eburneum and M. acuminata), Men *et al.*, 2003; (Dendrobium nobile), Muralidhar and Mehta, 1985; (Cymbidium longifolium), Seeni and Latha, 2000 (Vanda coerulea), Stewart and Kane, 2006 (Habenaria macroceratidis), and Zhang *et al.*, 1993 (*D. candidum*)]. Nagaraju *et al.* (2006) made use of 2% sucrose in KC and Nitsch medium while 3% sucrose was used in MS medium for the germination of *Cymbidium* hybrid. However, a higher sucrose concentration of 3% [Dendrobium hybrid (Lekha Rani *et al.*, 2005)] and 4.5% [Phalaenopsis (Young *et al.*, 2000)] has also been utilized for the purpose. Sharma and Tandon (1990) reported that 2 and 3% sucrose was optimal for germination of *Coelogyne punctulata* and *Cymbidium elegans* whereas a higher concentration of 7% was inhibitory. Gayatri and Kavyashree (2007), nevertheless, used sorbitol (3%) as the carbohydrate source for the asymbiotic germination of *Epidendrum radicans*. Sucrose and glucose are, in general, the best carbohydrate sources for the
Discussion

germination and seedling growth in orchids (Pathak et al., 2001). In a few terrestrial orchids, sucrose or a mixture of glucose and fructose greatly enhanced germination of embryos (cf. Mitra, 1987). Sucrose is not only by far the best form of carbohydrate as a source of energy but also required to maintain suitable osmolarity. The reason for sucrose being the best source of carbohydrate for germination of seeds could be that during sterilization of the medium in the autoclave it is broken down into more effectively usable sugars, glucose and fructose (cf. Gayatri and Kavyashree, 2007). However, synergistic effect of glucose and fructose was observed by Gayatri and Kavyashree (2007) and Gupta (2003). Some form of sugar is necessary, either in early stages to promote germination, or later to stimulate the development of protocorms. While a majority of the species can utilize a wide variety of sugars as carbon source, organic acids and L-series of sugars are of little value for the purpose. Certain species, although able to use many different sugars, do show some preferences. On the other hand, some species fail to germinate unless supplied with a specific sugar or combination of sugars in the medium. These differences could be due to specific requirements for selected sugars (Sharma and Tandon, 1990).

5.1.2.1.4 Activated charcoal

A common problem encountered during the in vitro culture of orchid tissues and organs is the high levels of phenolics released by tissues, which was combated by the use of activated charcoal in the medium, in the present study. The effect of AC on the adsorption of phenolics produced by the tissues and thereby leading to its positive effect was also stated by Yam et al. (1989). AC is also known to improve aeration. Growths similar to that on charcoal containing media was observed when seedlings of *Paphiopedilum* and *Phalaenopsis amboinensis* were grown on Pyrex glass wool alone or in combination with Nuchar C vegetable charcoal (cf. Yam et al., 1989). Another explanation could be that charcoal adsorbed 5-hydroxymethylfurfural which is produced by the dehydration of sucrose during autoclaving, which eventually lead to inhibition of growth (cf. Yam et al., 1989). In the present study, 0.2% AC was used for the purpose. AC was used at different concentration in *Cymbidium* (Nagaraju et al., 2006), *Cymbidium*
Discussion

lowianum (Nongdam et al., 2006; 0.2%), Dendrobium nobile (Men et al., 2003; 0.5%), Phalaenopsis (Young et al., 2000; 0.5%), and Vanda coerulae (Seeni and Latha, 2000).

Werkmeister (1970 a, b) first used charcoal to darken an asymbiotic culture medium to culture Cymbidium plantlets derived from shoot tip explants and to study root growth. In addition to being incorporated into media used for seed germination and seedlings, charcoal has also been added to formulations employed for the culture of orchid plantlets derived through tissue culture and for other plants (cf. Yam et al., 1989). In the present study, it was observed that in general, AC was beneficial only when the auxins and cytokinins were used together. This could perhaps be due to the capacity of AC to adsorb hormones and vitamins and thereby limit growth. We, therefore, suggest that it ought to be used with care in media with additives. However, in the cases where AC was not inhibitory it could be said that AC did not adsorb enough of any one of the component additive to inhibit growth; or if the component additive was adsorbed, it was probably not required, or in fact might be required only at the concentration at which it was left in the medium. Yam et al. (1989) echoed related views in the matter. They have also stated that the value of AC could be unpredictable as it could modify media by removal of components or inhibitors either present in the media or produced by the explant.

AC supported root formation and eventual seedling development in the current study. On a similar note, enhancement of root formation was reported in some orchid species but it might be partly attributed to the exclusion of light from the medium as suggested earlier by Yam et al. (1989). The observation in the case of Bletilla striata was also similar to the present study where graphite or charcoal powder enhanced the elongation of root tips (Yam et al., 1989). During the current investigations, AC (0.2%) had an inhibiting effect on the percentage of seed germination in both M and MS media compositions irrespective of the age of the seeds. In contrast to the present study, addition of AC assisted in stimulating growth in Cymbidium hybrids (Nagaraju et al., 2002) by causing an enhancement in the number of protocorms developed, an increase in the size of protocorms, and better root and root hair growth. Nongdam et al. (2006) achieved an increase in seed germination when the basal M medium was fortified with AC in C. lowianum. AC proved beneficial for promoting germination, early root formation and
subsequent seedling formation in *Epidendrum ibaguense* (Hossain, 2008) and *Cattleya aclandiae*, *C. grandulosa*, *Cattleyopsis lindenii*, and *Dendrobium parishii* (Buyun et al., 2004). Nagaraju et al. (2002) indicated that AC (3 g l\(^{-1}\)) in MS medium was superior with regard to protocorm number and root growth in *Cymbidium* hybrids when compared with KC and Nitsch media. In the current study, MS medium enriched with AC led to delayed protocorm development in immature seeds (16 wap). In consonance with the present results, Nongdam et al. (2006) reported that basal MS medium developed protocorms in *C. lowianum* seeds later than 13 weeks (obtained in M medium) and the culture failed to develop beyond the protocorm stage even on incorporation of AC in the medium.

### 5.1.2.1.5 Photoperiod

During the present study, 16/8 h L/D photoperiod was successfully used for seed germination as well as regeneration and maintenance experiments. Literature studies reveal varied reports on the effects of light/darkness on orchid seed germination and seedling development. The role of photoperiod in orchid seed germination is often overlooked and also not well understood for terrestrial species. Orchid seeds vary significantly in their requirement for light. The epiphytic and some terrestrial orchids germinate both in light and dark, although they appear to require light for shoot and root induction (Arditti and Ernst, 1984). According to Arditti et al. (1981), the germinating orchid seeds and developing seedlings vary in their tolerance, requirements, and response to light. Chung and Chung (1983) did not observe much variation in *Cymbidium densifolium* embryo germination under light/dark conditions, while Harvais (1973) and Stoutamire (1974) found light to be inhibitory to the germination of embryos in *Cypripedium reginae*. Similar inhibitory effect of light on germination was observed in *Paphiopedilum callosum* and *P. spicerianum* (Kano, 1965), and *Spiranthes odorata* (Lawrence et al., 1997). In some terrestrial species, even a pre-treatment with light has been shown to have an inhibitory effect on germination (cf. Pathak et al., 2001). Takahashi et al. (2000) reported successful seed germination in *Habenaria radiata* under light conditions. Similar to the present studies, 16/8 h L/D photoperiod was fruitfully provided for seed germination in *Aerides odorata* (Pant and Gurung, 2005), *Dendrobium aphyllum* (Talukdar, 2001), *Peristeria elata* (Bejoy et al., 2004), and *Vanda amesiana*.
Discussion

(Devi et al., 2006). A photoperiod of 14/10 h L/D regime was followed in *D. nobile* (Men et al., 2003) and *V. tessellata* (Bhadra et al., 2005) whereas a 12 h photoperiod was used for germination in *Aerides odorata, Dactylorhiza pierardii, Phaius tankervilliae,* and *Rhyncostylis retusa* (Singh et al., 2006), *Cymbidium lowianum* (Nongdam et al., 2006), and *Dendrobium* hybrid (Lekha Rani et al., 2005). Bhadra and Hossain (2004) successfully utilized the 10/10 h L/D regime in *Micropera pallida.*

In the current study, the effect of both 16/8 and 0/24 h L/D were tested and it was observed that, in general, photoperiod did not affect the germination percentage whereas 0/24 L/D regime advanced the onset of germination. However, Stewart and Kane (2007) reported that 16/8 h L/D photoperiod suppressed seed germination in *Habenaria macroceratitis.* They advocated the use of 0/24 h L/D photoperiod for maximum seed germination and most advanced protocorm development (emergence of first leaf); and stated that either the total absence of light or the total absence of darkness stimulated seed germination to relatively high percentages. Stewart and Kane (2006) studied the effect of 0/24, 16/8, 24/0 h L/D photoperiod for asymbiotic seed germination and 8/16, 12/12, 16/8 h L/D for seedling development and concluded that seeds of *Vanda* hybrids were subjected to three different photoperiods (8/16, 12/12, 16/8 h L/D) by Johnson and Kane (2007) whereas seeds of *Bletia purpurea* and *Cryptopodium punctatum* were subjected to either a 0/24 h or 16/8 h L/D photoperiod (Dutra et al., 2008, 2009). In contrast to the present study, Muralidhar and Mehta (1985) subjected the seeds of *Cymbidium longifolium* to continuous light conditions. A scrutiny of literature reveals that terrestrial orchids may have their own preference for a given light and/or dark treatment depending upon species. Katiyar et al. (1987) exposed the cultures of *C. punctulata* and *A. multiflorum* to 12 h photoperiod after the formation of protocorms. Some species prefer dark conditions while others may have better germination in light conditions (cf. Katiyar et al., 1987). However, cultures of *Vanda* hybrids incubated in dark showed the same percentage of germination, though the protocorms were achlorophyllous (Mathews and Rao, 1985 b). Similarly, Xu et al. (2001) in their studies on *D. chrysotoxum* concluded that light had no effect on embryo germination. *In vitro* seedlings cultured under 8/16 h L/D conditions produced the highest number of tubers per seedling. Final per cent of germination and protocorm development in *Habenaria macroceratitis* increased in the
Discussion

absence of light (0/24 h L/D). In the present study, it was found that dark conditions, though, promoted the onset of germination, light incubation was requisite for further development. Similarly, the results of Kauth et al. (2006) suggested that while darkness promoted seed germination, light enhanced further seedling development in Calopogon tuberosus. During the course of the present study, in addition to a 16/8 h L/D photoperiod, continuous dark (0/24 h L/D) period for initial 60 days was also utilized with success. In consonance with the current study, Sharma and Tandon (1987, 1990) followed suit in Coelogyne punctulata and Cymbidium elegans. Chang et al. (2004) subjected the seeds of D. lituifolium to dark conditions for the initial 20 days, later shifting them to light incubation. Germination percentage of Dactylorhiza majalis was increased by illuminating seeds with white light in 16 h photoperiods; optimal exposures (10–14 days) raised germination from 40 to 75%. Longer light treatments resulted in reduced germination percentage and smaller seedlings (Rasmussen et al., 1990). Seedling development in Platanthera integrilabia was enhanced by initial light compared to seedlings under continuous darkness. It was speculated that light exposure followed by darkness occurs naturally starting with the shedding of seeds from capsules to their immersion in a substrate where germination occurs. The beneficial effect of light was in support of light usage to stimulate germination and seedling development of temperate terrestrial orchids (Zettler et al., 1994). However, the seizure of development of Malaxis acuminata at spherule stage (Dhiman, 1999) and a delayed protocorm development of Pleione in the dark (Light and Macconil, 1990) implied that factors other than plant habitat play a significant role in influencing the light/dark requirements of seeds during the course of germination.

In the current study, the effect of light and dark incubation (16/8 or 0/24 h L/D photoperiods) on the percentage of germination varied with the growth adjunct in the nutrient medium and seeds germinated equally well under both the conditions; though dark period led to an earlier onset. However, presence of light (16/8 h L/D) was obligatory for the growth and development of the germinating entities. In a similar way, Johnson and Kane (2007) reported that the effect of different photoperiods (8/16, 12/12, 16/8 h) was not conclusive of a pattern in Vanda hybrids. They also stated that Vanda hybrid seeds did not develop as rapidly as those cultured in light conditions. On a similar
Discussion

note, Sharma and Tandon (1987) showed that light did not inhibit germination in the species tested i.e., *Cymbidium elegans*, *Coelogyne prolifera*, *C. cristata*, *C. porrecta*, *Aerides multiforum*, *Sarcanthus pellidus*, *Bulbophyllum cosmosus*, and *Thunia alba*. The protocorms of *A. multiforum*, *S. pellidus*, *T. alba*, *B. cosmosus*, *C. porrecta*, and *C. prolifera* were insensitive to light; they were initially white and remained white upon illumination also. On the other hand, protocorms of *Cymbidium elegans* and *Coelogyne cristata* were green even in dark but showed pronounced growth when illuminated. In accord with the current investigations, Dutra et al. (2008) earlier recorded *Bletia purpurea* seeds to germinate regardless of the photoperiod conditions (0/24 h or 16/8 h L/D); however, seedlings were obtained only in 16/8 h L/D conditions. Presently, dark conditions, in general, advanced the onset of germination in *M. acuminata*; the species grows under shady conditions. According to Stewart and Kane (2006) seed germination of many terrestrial orchids has been found to be inhibited by incubation in light. Light also plays an important role in in situ seed germination. Stewart and Kane (2006) demonstrated no effect of photoperiod on initial asymbiotic seed germination of *Habenaria macroceratitis*. However, a significant effect on subsequent protocorm growth and morphological development was shown. *H. macroceratitis* typically inhabits heavily shaded floors of hammock habitats in Central Florida. The seeds inhabiting shaded floors were not exposed to large quantities of red light, but in fact were exposed to far-red light. Seeds exposed to far-red light would convert phytochrome into the Pr form, thus inhibiting germination. Given that far-red light is known to inhibit seed germination and cool white fluorescent tubes emit large amounts of red light, so the highest per cent seed germination occurred in a 0/24 h L/D photoperiod (complete darkness) (cf. Stewart and Kane, 2006). Earlier, Stoutamire (1974) proposed that species which colonize open or partially shaded habitats are more likely to benefit from light at an early stage of germination. The inhibitory effect of light on germination has been described as a part of protective mechanism, which makes it impossible for the seedlings to develop at the surface soil where they would be subjected to drying.
Discussion

5.1.2.1.6 Organic adjuncts

A large number of complex growth supplements are routinely employed in order to enrich the culture media for orchid seed germination. Some such common as well as uncommon additives are apple juice, banana homogenate (BH), beef extract, casein hydrolysate (CH), coconut water (CW), extract of silkworm pupae, fish extract, honey, peptone (P), potato extract, tomato juice, and yeast extract (YE) (Arditti, 1967). The growth promoting effect of these has been credited to their organic nitrogen (as amino acids and amides), minor elements, and vitamin constituents (Raghavan, 1976). In the current study, the effect of CH/CW/P/YE was assessed on seed germination and subsequent seedling development.

5.1.2.1.6.1 CW

Currently, CW was effectively used at various concentrations (5, 10, and 15%) for germination (Fig. 393). Enrichment of the medium with CW (liquid endosperm of Cocos nucifera) has been utilized since long for obtaining growth and organ differentiation in orchid cultures. CW induces division of the otherwise non-dividing cells (Goh et al., 1975) and multiplication of protocorms (Intuwong and Sagawa, 1973). CW contains inorganic ions, nitrogenous compounds, vitamins, sugar, amino acids and related substances besides being rich in certain plant hormones (auxins, cytokinins, 1,3-diphenylurea, and gibberellins). Its stimulatory effect has been attributed partly to zeatin riboside content (cf. Devi et al., 2006; cf. Bejoy et al., 2004). The kinetin content in coconut water might be the probable cause of growth enhancement (cf. Lekha Rani et al., 2005). Intuwong and Sagawa (1973) reported that CW promoted early protocorm differentiation and vigorous seedling development. Similarly, Kitsaki et al. (2004) positively tested coconut milk-enriched or a pineapple-enriched medium for the germination of Ophrys delphinensis and O. spurneri. Earlier workers have also successfully used this growth adjunct for the germination of Dendrobium hybrid (Lekha Rani et al., 2005), Peristeria elata (Bejoy et al., 2004), Vanda coerulea (Seeni and Latha, 2000). Enhancement of foliar growth by the addition of coconut water has also been reported (cf. Lekha Rani et al., 2005). Gupta (2003) reported the advancement of the onset of morphogenetic changes leading to seedling development in Cymbidium
Discussion

*M. acuminata* germinated and formed protocorms on M and MS media enriched with coconut water. CW at 10% concentration proved the best for enhancing the germination percentage as well as for seedling development. Earlier, Bejoy *et al.* (2004) advocated the use of CW at 10% concentration in *Peristeria elata*. Sheelavantmath *et al.* (2000) also showed the formation of PLBs in *Geodorum densiflorum* when supplied with 10% CW. However, Lekha Rani *et al.* (2005) reported 20% CW followed by 10% to be optimal for early protocorm differentiation and better seedling formation in *Dendrobium* hybrid whereas 15% was found to be beneficial for maximum seedling growth in *Peristeria elata* (Bejoy *et al.*, 2004).

Fig. 393 The effect of coconut water, nutrient medium and stages of seed development on in vitro seed germination in *Malaxis acuminata*

5.1.2.1.6.2 P

A water soluble protein hydrolysate with high amino acid content, P (0.1%) was beneficial for inducing leaf formation and pseudobulb multiplication; though germination percentage was reduced during the present studies. In accord with the current study, P impaired germination in *Dactylorhiza maculata* (Van Waes and Debergh, 1986) and *Vanda tessellata* (Roy and Banerjee, 2002), and proved ineffective in *Calanthe discolor*.
However, P has been shown to be beneficial for better germination in *Cattleyopsis lindenii* and *D. parishii* (Buyun *et al.*, 2004), *Epidendrum ibaguense* (Hossain, 2008), *Malaxis acuminata* (Gupta, 2003), *Vanda* hybrid (Johnson and Kane, 2007), *V. amesiana* (Devi *et al.*, 2006), and *V. teres* (Sinha and Roy, 2004); accelerated organogenesis in *Cymbidium eburneum* (Gupta, 2003); and for an increase in protocorm multiplication in *C. macrorhizon* (Vij and Pathak, 1988) and *Dactylorhiza hatageria, Eulophia dabia, M. acuminata,* and *Saccolabium papillosum* (Sharma, 2009). It promoted the formation of healthy seedlings in *V. amesiana* (Devi *et al.*, 2006). Addition of P (1 g l\(^{-1}\)) with cytokinin zeatin (0.72 \(\mu\)M) and auxin NAA (1.34 \(\mu\)M) to the culture medium significantly increased the growth parameters of seedlings of *Dactylorhiza incarnata* subsp. *serotina, Dactylorhiza maculata* subsp. *maculata, Liparis loeselii* (Vejsadová, 2006). The above data indicate that efficacy of P varies with the species and the developmental stage of the germinating entities. According to Arditti *et al.* (1982), the discordant effect may be attributed to the source and batch of P supply.

5.1.2.1.6.3 CH

An amino acid complex, CH has been extensively utilized as a growth adjunct in orchid seed germination media. During the current investigation, CH (0.1%) supported only marginal germination per cent in *Malaxis acuminata*, however, pseudobulb multiplication was induced. In consonance with the current results, CH impaired germination in *Saccolabium papillosum* (Znaniecka *et al.*, 2005). It was, similarly, shown to be inhibitory to organogenesis and rhizogenesis in *Cymbidium eburneum* and *M. acuminata*, respectively (Gupta, 2003) and germination in *Herminium lanceum* (Mahant, 1991). However, the beneficial role during germination and seedling formation has been demonstrated in *Aerides multiflora, Rhynchostylis retusa, Saccolabium papillosum,* and *Vanda testacea* (Vij *et al.*, 1981). CH was shown to be requisite for the germination in *Cypripedium calceolus* and *Epipactis helleborine* whereas it was not necessary in *Listera ovata* and *Dactylorhiza maculata* (Van Waes and Debergh, 1986). CH promoted the formation of healthy seedlings in *V. amesiana* (Devi *et al.*, 2006). In contrast to the current study, CH was shown to be constructive in germination and callus mediated protocorm multiplication and earlier seedling formation in *M. acuminata* (Sharma, 2009).
The variable effect of CH during germination may be credited to its compositions, which vary with the extent of hydrolysis of the nitrogenous substances present in the casein (Arditti et al., 1982).

5.1.2.1.6.4 YE

YE is also an essential source of reduced nitrogen and has been efficiently utilized in germination and proliferation in various orchid species. In the current investigations, the use of YE (0.1%) reduced germination and leaf and root formation; though pseudobulb formation and multiplication was supported. In consonance with present results, it was proven to be inhibitory to organogenesis in Cymbidium eburneum and rhizogenesis in M. acuminata (Gupta, 2003), and shoot development in Cattleya (Kusumoto, 1979). In contrast, YE has been demonstrated to be favourable for germination in several orchids (Mahant, 1991; Pathak, 1989; Salwan, 1992; Kaur, 1996). Similarly, YE was found to be beneficial for germination and protocorm multiplication in Eulophia dabia and M. acuminata, and for germination and seedling development in Habenaria commelinifolia (Sharma, 2009) whereas YE was shown to be inhibitory to PLB development in Vanda amesiana (Devi et al., 2006).

5.1.2.1.7 Plant growth regulators

Plant growth regulators (PGRs) are usually the key to control the plant growth and development in vitro. Early attempts at regeneration of plants from cells or tissues in vitro failed until natural substances, containing what we now recognize as auxins and cytokinins, were included in the medium. The pioneering work of Skoog and Miller (1957) established the critical role of auxins and cytokinins. The balance of auxins and cytokinins determines the type of growth. In the present study, M medium was fortified with various growth regulators, alone or in combination, to mimic the role of mycorrhizal association during germination of orchid seeds in nature. That the exogenous supply of growth regulators mimics the role of mycorrhizae has been suggested by earlier workers (Devi et al., 2006; Pathak et al., 2001).
5.1.2.1.7.1 Cytokinins

The function of cytokinins has been tested in various orchids and they respond variously; the germination percentage being enhanced or impaired or remaining unaffected (Arditti and Ernst, 1984; Pahtak et al., 2001). During the current investigations, the effect of BAP and KN was assessed on the germination potential.

5.1.2.1.7.1.1 BAP

During the current study, it was observed that BAP fortified medium though supported the formation of leaf and pseudobulb multiplication at 6.6, 8.8, 13.2 and 20.0 μM concentrations; however it was not instrumental in promoting high per cent germination. Similarly, Devi et al. (2006) reported that BAP suppressed root formation though pseudobulbs were developed at higher doses of the growth regulator; nevertheless, it impaired leaf formation in Vanda amesiana. In consonance with the present studies, BAP (2.5 mg l⁻¹) promoted protocorm multiplication leading to the formation of PLB clusters in V. tessellata (Bhadra et al., 2005). In the current results, the effect of AC was variable with the different concentrations of BAP. However, conflicting report of regeneration, multiplication and root formation in Oncidium species in the presence of BAP (2.0 mg l⁻¹) was given by Kalimuthu et al. (2007). BAP (1.0 mg l⁻¹) was, however, shown to be more beneficial in promoting germination in Epidendrum ibaguense by Hossain (2008). BAP (0.5-2.0 mg l⁻¹) has proved effective for seed germination and seedling development in Aerides odorata (Pant and Gurung, 2005). During the current investigations, the presence of BAP did not advance the germination onset while Nagaraju et al. (2002) reported earliest swelling of seeds of Cymbidium hybrids in BAP supplemented medium. Nongdam et al. (2006) also showed its detrimental effects in both M and MS media and did not support development beyond spherule stage. In contrast to the current report, the enhancing effect of BAP on germination and seedling formation in Dactylorhiza incarnata, D. maculata, and Liparis loeselii was demonstrated (Vejsadová, 2006).
The presence of KN invariably reduced the per cent seed germination in the present study. Lower concentration of 4.4 μM gave better germination frequency under light conditions and also supported pseudobulb formation and multiplication. In consonance with the above results, Arditti and Ernst (1984) opined that germinating orchid seeds were more sensitive to higher cytokinin levels than the protocorms, indicating their lower or no requirement for cytokinins. The seeds of *Malaxis acuminata* germinated successfully in media lacking cytokinins, it appears that they were cytokinin autonomous. Mercier and Kerbauy (1991) and Van Waes and Debergh (1986), similarly, demonstrated that *Epidendrum fulgens* and *Dactylorhiza maculata* and *Listera ovata* seeds, respectively germinated best in medium lacking a cytokinin. Mercier and Kerbauy (1991) opined that orchid species that do not require an exogenous cytokinin source for germination are cytokinin autonomous as they contain high endogenous cytokinin levels. Talukdar (2001) reported that KN stimulated shoot multiplication from the protocorms of *Dendrobium aphyllum*, but growth in cultures was rather slow. They reported considerable number of roots forming in *D. aphyllum* in the medium supplemented with KN, whereas in the present study KN did not promote any rooting. Sharma (2009) reported positive effect of KN on seed germination and accelerated seedling formation in *Habenaria commelinifolia* whereas lower germination frequency was observed in *Dactylorhiza hatageria* and *Saccolabium papillosum*; however, in *Eulophia dabia*, its effect was subjective to the presence of light. In *D. hatageria*, it led to slightly earlier onset of germination and promoted leaf differentiation.

**5.1.2.1.7.2 Auxins**

Orchid seeds are known to contain very low amounts of auxins (cf. Arditti and Ernst, 1984). Their auxin requirements for germination in nature are augmented by the fungal partner in mycorrhizal association (Hayes, 1969). Presently, the effect of IAA, IBA, and NAA was assessed singly or in combination with the cytokinins.

**5.1.2.1.7.2.1 IAA**

When the medium was enriched with IAA, as many as 60% seeds germinated under light conditions only at lower concentration of 4.4 μM. However, IAA, in general,
supported morphogenesis only till the protocorm stage. Pseudobulbs were formed only at 8.8 μM. In disagreement with the above consequences, Manrique et al. (2005), however, reported impaired germination frequency in Comparettia falcata in IAA supplemented medium. In contrast to the above results, Devi et al. (2006) reported IAA to favour PLB multiplication in Vanda amesiana; while root and leaf formation was favoured only at 0.1 and 2.0 mg l⁻¹; however, it proved ineffective (0.5 mg l⁻¹) and detrimental to growth (1.0 mg l⁻¹) at lower concentrations in consonance with the current investigations. In the present study, IAA was not found to have any positive impact on the seed germination percentage and differentiation of protocorms occurred at higher concentrations of 6.6 and 8.8 μM. However, Talukdar (2001) reported stimulatory effect of IAA (10.0 mg l⁻¹) on seedling growth and protocorm differentiation and leaf formation in Dendrobium aphyllum. Favourable effects of IAA on germination and differentiation on Habenaria commelinifolia and Malaxis acuminata, and early development of protocorms and leaf primordia in Dactylorhiza hatageria were reported (Sharma, 2009).

5.1.2.1.7.2.2 IBA

The presence of IBA in the medium was unable to match up to the germination percentage shown by the basal medium in the current study. However, leaf formation and pseudobulb development occurred at a higher concentration of 8.8 μM. Similarly, IBA supplemented medium supported leaf formation in Vanda amesiana (Devi et al., 2006); IBA at 0.5 mg l⁻¹ also supported leaf formation. In contrast to the current results, IBA supplemented medium supported cent per cent germination in Bulbophyllum careyanum and Dendrobium chrysotoxum, promoted protocorm multiplication in D. chrysotoxum, and favoured luxuriant seedling growth in B. careyanum and D. chrysotoxum (Kher, 1999). IBA induced seed germination in Cymbidium lowianum and healthy seedlings were obtained in 32 weeks in M medium; while IBA in MS medium was not conducive for germination (Nongdam et al., 2006). IBA improved the germination frequency but remained ineffective for subsequent morphogenetic events in the germinating entities in Habenaria commelinifolia (Sharma, 2009).

5.1.2.1.7.2.3 NAA

During the investigations at hand, NAA at 8.8 μM showed germination percentage of 63.5% which was comparable to basal M medium in the presence of AC.
NAA at 6.6 µM was beneficial in supporting 47% seed germination, pseudobulb formation and rooting. However, contrary to our results, *Vanda tessellata* seeds formed roots in the medium containing IAA (1.0 mg l\(^{-1}\)) (Bhadra *et al.*, 2005). NAA promoted seedling growth in *V. amesiana* but its effect on PLB induction was dose specific (Devi *et al.*, 2006). In resemblance with the present study, Devi *et al.* (2006) stated that additional use of NAA favoured PLB multiplication of *V. amesiana* but their effect on leaf and root formation varied with the quality and concentration of the PGRs. In the current study, NAA was more effective at lower concentration, though an odd result was shown by NAA at 8.8 µM under light conditions (63% germination). On a similar note, lower concentration of NAA was reported to be beneficial while higher concentration reduced the seedling growth in *Cattleya* and *Denrobium* (Vajrabhaya and Vajrbhaya, 1976) and *Pachystoma senile* (Sood, 1984). In a similar way, Pant and Gurung (2005) reported that lower concentration of NAA was more useful than its respective higher concentration in inducing seed germination of *A. odorata*. In consonance with the present results, Tang *et al.* (2005) also found NAA (0.5 mg l\(^{-1}\)) to produce highest number of roots and root length in *Dendrobium candidum*. However, stimulatory effect on protocorm differentiation and seedling formation was reported in NAA supplemented medium (5.0 and 10.0 mg l\(^{-1}\)) in *D. aphyllum* (Talukdar, 2001). NAA reduced the germination frequency but enabled earlier leaf differentiation in *Dactylorhiza hatageria* (Sharma, 2009). In the present study, root formation was stimulated by NAA (6.6 µM) enriched medium. Presence of NAA supported efficient seed germination and rapid protocorm development in *Cymbidium lowianum* in addition to proving best for producing higher number of roots and rapid root initiation (Nongdam *et al.*, 2006). Gupta (2003) also reported early protocorm development in *Cymbidium eburneum*. Chang *et al.* (2004) observed that NAA (0-0.5 mg l\(^{-1}\)) was largely beneficial for protocorm multiplication in *Dendrobium lituiflorum*. In the presence of NAA, dark conditions were unable to enhance the germination potential of the seeds in the present study. On a similar note, in *Eulophia dabia*, the efficiency of NAA varied with light conditions; growth and seedling development was promoted when the cultures were provided with a 12 h photoperiod whereas dark incubated cultures failed to develop beyond the spherule stage (Sharma, 2009).
**5.1.2.1.7.3 Cytokinins and auxins**

In the combinations containing BAP and IAA, all concentrations reduced the germination response; in general, the growth was arrested at the protocorm stage in the current investigations. Under light conditions the combination caused chlorophyllous protocorm formation and their multiplication in the present study; however, no seedlings were formed. However, in a study by Bhadra *et al.* (2005) it was found that multiplication of protocorms along with simultaneous elongation of seedlings occurred in *Vanda tessellata*.

In the present study, the addition of both BAP and NAA led to a reduced germination percentage; though protocorms were developed. When 2,4-D was supplemented to the medium either singly or in combination with BAP, it led to a remarkable reduction in the germination of *Epidendrum ibaguense* (Hossain, 2008). However, multiplication of protocorms as well as elongation of seedlings occurred on BAP (2.0 mg l⁻¹) and NAA (2.0 mg l⁻¹) enriched medium in *V. tessellata* (Bhadra *et al.*, 2005). Similar observation was reported in *Cypripedium macranthos* (Shimura and Koda, 2004). Pant and Gurung (2005) stated that use of BAP (2.0 mg l⁻¹) and NAA (1.0 mg l⁻¹) proved to be the best for seed germination among all other hormone combinations for *Aerides odorata*; lower concentration of the two growth regulators was more effective than their respective higher concentration. Contrasting results were also obtained in *Dendrobium candidum* where BAP (1.0 mg l⁻¹) and NAA (0.1 mg l⁻¹) were found to be very beneficial to protocorm differentiation and propagation (Tang *et al.*, 2005). The best response of seedling growth in *Vanda coerulea × Ascocentrum auranticum* was observed in medium supplemented with KN (2.3 μM) and NAA (0.5 μM) (Kishor *et al.*, 2006).

Presently, comparatively less efficient response of plant growth regulators on germination and the formation of seedlings than the basal medium could perhaps be because of their effect on physiological processes or interaction between the growth regulators. Related views were presented by Pant and Gurung (2005) in *Aerides odorata*. In the present case, 100% seed germination was unattainable which was most likely due to inherent non-viability of the seeds. Pant and Gurung (2005) was, likewise, not able to attain cent per cent germination for *A. odorata*. It appears that the results of experiments related to the effect of PGRs on orchid seed germination and seedling development are
Discussion

inconsistent and inconclusive. Earlier Arditi and Ernst (1984) attributed such incoherences to hormonal interactions; other inclusions in the media; culture conditions; variable physiological responses and requisites of species and genera; different forms/batches of the growth regulator used; wide range of growth regulator concentrations; and variations in the age of seeds. The irregularities in results with growth regulators suggest a species-specific requirement of the growth regulators. In the current study, basal MS medium proved the best (97%) for highest seed germination. Immature seeds of *Satyrium nepalense* were germinated on MS, KC, and KC modified Morel medium; MS medium was found to be the best for seed germination (86.7%) and protocorm development (Mahendran and Narmatha Bai, 2009). MS basal medium at half-strength was effective for the development of the hybrid seedlings of *Vanda coerulea × Ascoctenrum auranticum* followed by VW and KC media (Kishor et al., 2006). Our observation that the percentage of germination and seedling growth varies with the nutritional regime was in agreement with comparable earlier findings (Singh et al., 2006 b). Singh et al. (2006 b) reported a better efficacy of Nitsch medium for seed germination of seeds of *Aerides odorata, DendrobiumDactylorhiza pierardii, Phaius tankervilliae*, and *Rhynchostylis retusa* as compared to B5, KC, MS and VW media. It may be concluded that no single ingredient as such stimulated germination or seedling growth in orchids; it was rather an interaction between the additives and the compounds of media. Lekha Rani et al. (2005) also made a similar observation. Nagaraju et al. (2002) attributed variation of responses of *Cymbidium* hybrid seeds in different media to the variation in the amount of salts, amino acids, vitamins, and minerals.

5.1.2.2 Regeneration

5.1.2.2.1 Pseudobulb

Due to en bloc elimination of the orchid species in certain areas, an efficient strategy has become necessary for salvaging and multiplying these for adequate conservation purposes. Propagation through seed germination in nature has a long juvenile period before flowering. Plant tissue culture offers opportunities to conserve and multiply such threatened and over-exploited species. Presently, regeneration potential of segments (0.5 cm long) obtained from *in vivo* sourced pseudoblubs (4.0 cm long) was
successfully tested in *Malaxis acuminata*. Earlier, pseudobulbs have been productively used for regeneration purposes in *Bulbophyllum careyanum* (Vij et al., 2000), *Cattleya maxima* and *Cymbidium* hybrid (Nagaraju et al., 2003), *Dendrobium candidum* (Zhao et al., 2007), *D. chrysanthum* (Vij and Pathak 1989), *D. macrostachyum* (Pyati et al., 2002), *Eulophia hormusjii* (Vij et al., 1989), *Geodorum densiflorum* (Sheelavantmath et al., 2000), *M. acuminata* and *Cymbidium eburneum* (Gupta, 2003), and *Micropera pallida* (Bhadra and Hossain, 2004).

During the current study, M medium with or without growth regulators was utilized for regeneration in *Malaxis acuminata* pseudobulbs. Likewise, Decruse et al. (2003 a) and Vij et al. (1989) used M medium in the presence or absence of growth regulators in *Vanda sathulata* and *E. hormusjii*, respectively. However, various reports of the use of KC [Nagaraju et al., 2003, *Cattleya maxima* and *Cymbidium* hybrid; Sheelavantmath et al., 2000, *G. densiflorum*], MS [Bhadra and Hossain, 2004, *Micropera pallida*; Nagaraju et al., 2003, *Cattleya maxima* and *Cymbidium* hybrid; Pyati et al., 2002, *D. macrostachyum*; Sheelavantmath et al., 2000, *G. densiflorum*; Vij and Pathak, 1989, *D. chrysanthum*; and Zhao et al., 2007, *D. candidum*], and VW [Vij et al., 2000, *B. careyanum*] media are available. Nagaraju et al. (2003), in addition to MS and KC also made use of Nitsch and Nitsch, Street, White, and Gamborg media for regeneration in *Cattleya maxima* and *Cymbidium* hybrid. Full strength M medium was utilized in the present regeneration studies; however, half strength MS medium was used for the purpose by Zhao et al. (2007). Pyati et al. (2002) used both full and one-fourth strength MS medium for regeneration studies in *D. macrostachyum*.

In the current taxon, sucrose was added at 2% concentration to the medium. Precedingly, sucrose has been added at 2% concentration by Decruse et al. (2003 a), Sheelavantmath et al. (2000), Vij and Pathak (1989), Vij et al. (2000), and Zhao et al. (2007) in consonance with the present results. However, 3% sucrose was added to the medium by Pyati et al. (2002) whereas Nagaraju et al. (2003) added 2-3% sucrose depending on the nutrient medium.

During the course of the current study, pseudobulb sections failed to respond in the basal M medium showing that presence of BAP was obligatory for activating meristematic activity; they responded in BAP (8.8 and 22.0 μM) treated cultures with
Discussion

Shoot formation. In agreement with our study, the explants did not respond in the growth regulator-free medium and chemical stimulus of growth regulators was essential in *Bulbophyllum careyanum* (Vij et al., 2000), *Dendrobium candidum* (Zhao et al., 2007), *D. chrysanthum* (Vij and Pathak, 1989), *D. macrostachyum* (Pyati et al., 2002), and *Vanda* Poepoe Diana (Anirudham and Nair, 2006). However, Sheelavantmath et al. (2000) reported positive response in MS medium whereas basal KC medium failed to induce a caulogenic response in *G. densiflorum*.

In the present investigation, explants invariably regenerated via shoot bud formation. Presently, BAP (22.0 μM) was found to be the best for shoot bud induction and shoot elongation. Shoot bud response was, likewise, observed in the presence of BAP alone [ *Cattleya maxima* and *Cymbidium* hybrid (Nagaraju et al., 2003; 0.5 mg l⁻¹); *D. candidum* (Zhao et al., 2007), *G. densiflorum* (Sheelavantmath et al., 2000; 5.0 μM)], or in combination with NAA [ *D. candidum* (Zhao et al., 2007; 0.8-2.0 and 0.4-1.5 mg l⁻¹, respectively), *G. densiflorum* (Sheelavantmath et al., 2000; 1.0 mg l⁻¹ each), Micropera pallida (Bhadra and Hossain, 2004; 2.0 mg l⁻¹ each)], and IAA and YE [ *D. chrysanthum* (Vij and Pathak, 1989)]. BAP or KN was utilized by Pyati et al. (2002) in *D. macrostachyum* (4.44 and 8.88 μM, respectively) and Vij et al. (2000) in *B. careyanum* (2.0 mg l⁻¹) leading to multiple shoot buds in the latter case. However, Zhao et al. (2007) showed KN to have the lowest induction efficiency compared to BAP in *D. candidum*. CW (5%) was used for the purpose in *D. macrostachyum* with its increased concentration leading to higher number of shoot buds per explant (Pyati et al., 2002).

In the current study, rooting was induced in the shoots on NAA fortified medium (2.2 μM). NAA was successfully utilized for root induction in *B. careyanum* (Vij et al., 2000) and *G. densiflorum* (Sheelavantmath et al., 2000; 1.0 μM) and it was used in combination with IAA in *D. candidum* (Zhao et al., 2007; 1.0 mg l⁻¹ each). However, Bhadra and Hossain (2004) induced rooting with IAA (4.4 μM) in *Micropera pallida* while Sheelavantmath et al. (2000) reported failure of auxin (IBA and IAA) containing medium to induce rooting in *G. densiflorum*.

During the present investigation, the pseudobulb segments were placed in the upright orientation (with the basal i.e., proximal end cut surface touching the surface). The orientation appeared to interact with polarity to affect shoot regeneration. Earlier,
Gupta (2003) stated that their regeneration potential was markedly influenced by their position on the donor axis. In a similar way, Zhao et al. (2007) observed that if the explants were incubated in an upright position on the medium, shoots were induced more efficiently in *D. candidum*; for explants with the same position, the morphogenetic capacity of explants incubated in the upright orientation was higher than those in the inverted orientation.

The ability of *Malaxis acuminata* pseudobulb segments to regenerate shoot buds and ultimately form seedlings is promising and indicates their utility in micropropagating the species. It can be successfully employed for the rapid multiplication of identical genotypes in orchids by suitably adjusting the nutrient environment. Various tissues and/or organs can be induced to proliferate in vitro in a large number of species which implies that the latent meristematic activity can be activated under controlled conditions and this leads us to suggest that the pseudobulbs can be used for regenerating *M. acuminata* and potentially other pseudobulbous orchids (Vij et al., 1989).

### 5.1.2.2.2 Culture multiplication

Ever since, Morel (1964 a, b) demonstrated the ability of protocorms/PLB slices to regenerate large clumps of PLBs/daughter PLBs when sub-cultured on a fresh medium, protocorms/PLB sections have been fruitfully utilized to multiply orchid cultures (Kanase and Takano, 1995, Lam et al., 1991; cf. Mathews and Rao, 1985). Even the whole PLBs have been reported to be capable of generating daughter PLBs directly or indirectly through callusing (Vij et al., 1981 a). Stewart (1989) traced the proliferations in the single cell in the epidermal layers and suggested that they were embryogenic at explanting and did not require de-differentiation multiplication without excessive cellular destabilization during early stages in the life cycles. The extent of proliferation was, however, markedly influenced by the chemical stimulus in the nutrient pool. The organ differentiation depends on the genetic and physiological activities of the species (cf. Katiyar et al., 1987).

In consonance with the present conclusions, Mitra (1987) had earlier pointed out that in vitro culture of several orchids produced proliferating tissues which gave rise to nodular bodies each with a growing point which in turn often formed seedlings. Normally
the orchid embryos directly produce protocorms, from which the seedlings develop. Immature embryos, unlike full-term embryos are preferably selected to form unorganized growth in the initial stage of germination followed by organization of growing points in their unorganized growth leading to protocorm formation. Some embryos of *Dendrobium* *fimbriatum* formed proliferating tissue which was attached with protocorm differentiation while the rest developed directly into protocorms and seedlings (cf. Mitra, 1987).

In the present study, M as well as MS media were used with the latter being more productive for healthy seedling, pseudobulb multiplication and PLB formation. MS medium was similarly used for these purposes in *Aerides crispmum* (Sheelavantmuth et al., 2005), *A. rosea*, *Renanthera imshootiana*, and *Renandes* (Sinha et al., 2001), *Cymbidium aloifolium* (Hossain et al., 2008), *C. aloifolium* and *Dendrobium nobile* (Nayak et al., 2002), *Dendrobium* hybrids Sonia 17 and 28 (Martin et al., 2005), *Dendrobium* Sonia 17 (Sheela et al., 2004), *D. huoshanenese* (Luo et al., 2003), *Ipsea malabarica* (Martin and Madassery, 2005, Martin and Pradeep, 2003), *Otocilus alba* (Mukhopadhyay and Roy, 1994), *Phalaenopsis* (Young et al., 2000), *Pleione formosana* (Lu, 2004), and *Vanda coerulea* (Seeni and Latha, 2000). However, half strength MS medium was put to use in *D. caudacum* (Jiang et al., 2003) and *D. candidum* (Liu and Zhang, 1998). Lu (2004), Martin and Pradeep (2003) and Sinha et al. (2001) also used half strength MS medium for PLB proliferation. Various workers have utilized other media, namely B5 (*D. candidum* (Liu and Zhang, 1998)), Hyponex (*Phalaenopsis* (Young et al., 2000)), KC (*A. multiflorum* (Katiyar et al., 1987), *Coelogyne punctulata* and *Cymbidium elegans* (Sharma and Tandon, 1990), *D. aphyllum* (Talukdar, 2001), *Peristeria elata* (Bejoy et al., 2004), *Phalaenopsis* (Young et al., 2000), Lindemann (Young et al. (2000) (*Phalaenopsis*)), M (Bejoy et al. (2004) (*Peristeria elata*)), Phytamax (Mukhopadhyay and Roy (1994) (*Otocılıus alba*)), and VW (Sheela et al. (2004) (*Dendrobium* Sonia 17), Montri et al. (2008) (*Grammatophyllum speciosum*), Young et al. (2000) (*Phalaenopsis*)) media.

Both BH (60%) and CW (10%) were tested during the study on *Malaxis acuminata* and the former was found to be fruitful in terms of healthy seedling development and formation of pseudobulbous shoots, with the latter being inefficient. In the present study, the protocorms multiplied and developed into PLBs followed by PLB
Discussion

Very few PLBs formed plantlets in BH and CW enriched medium. Substances similar to cytokinin, auxin, gibberellin have been reported to be present in BH (Arditti, 1979). The growth promoting effect of BH was reported to be ‘synergistic interaction’ of various factors including buffering effects which help in maintaining the optimum pH leading to easy availability of iron for the growth of protocorms (cf. Talukdar, 2001). In consonance with the present study, BH has been successfully utilized for the purpose in Cypripedium (Talukdar, 2001), Dendrobium caudacum (Jiang et al., 2003), D. candidum (Liu and Zhang, 1998), and Vanda coerulea (Seeni and Latha, 2000). Contrary to the present study, Bejoy et al. (2004) used CW (10%) to form seedlings from protocorms of Peristeria elata. Similarly, CW has been efficiently used in Dendrobium hybrid (Sheela et al., 2004; 7.5-15%, along with BAP), Dendrobium Sonia 17 (Sheela et al., 2004; along with BAP and NAA/IAA), D. caudacum (Jiang et al., 2003; along with AC), and V. coerulea (Seeni and Latha, 2000; 30%, along with NAA). The utility of CW in activating multiplication of meristematic loci has been recorded in Ascocenda, Vanda and Renanthera (cf. Arditti and Ernst, 1993) and a variety of other hybrids and species (Lee et al., 1995, 1996). CW was shown to be beneficial in inducing proliferation and rapid PLB multiplication in Bulbophyllum careyanum and Cymbidium hybrid (Kher, 1999). Its benign effect has been variously attributed to sugar and cytokinin (Lee et al., 1995) and auxin and gibberellin (George and Sherrington, 1984) like components. In the present study, BH containing medium fruitfully induced rooting in the shoots. Likewise, root induction was reported in BH (35 g l⁻¹) and NAA (1.08 μM) fortified medium in V. coerulea (Seeni and Latha, 2000). Coconut water, when supplied at 10–20% resulted in a significant increase in the number of PLBs formed per PLB segment of hybrid Cymbidium Twilight Moon ‘Day Light’ (Silva et al., 2006). Coconut water was also utilized for PLB induction from protocorms in A. crispum at 5, 10, and 15% concentration (Sheelavantmath et al., 2005). Montri et al. (2008) indicated that different mixtures of banana pulp, pineapple pulp, tomato pulp and Jew’s Ear mushroom extract could promote protocorm development into seedlings in Grammatophyllum speciosum.

The effect of different plant growth regulators on protocorm multiplication, PLB proliferation and seedling development could not tested in the current study. However, plant growth regulators have been made use of by various earlier workers i.e. BAP (44.4
Discussion

μM) for PLB multiplication and KN (6.97 μM) for shoot formation therefrom (Martin et al., 2005), BAP alone (5.0 mg l⁻¹) for PLB multiplication (Hossain et al., 2008), BAP (13.3 μM) for PLB formation from axillary buds and KN for axillary bud proliferation (Martin and Madaserry, 2005), BAP along with KN, zeatin and NAA for shoot formation (Nayak et al., 2002), KN (1.5 mg l⁻¹) for storage of shoots up to 14 months without subculture in Ipsea malabarica (Martin and Pradeep, 2003), 2,4-D and TDZ (0.5 mg l⁻¹ each) for higher proliferation rate, and 2,4-D (1.0-5.0 mg l⁻¹) and TDZ (0.1-0.5 mg l⁻¹) for better totipotency (Lu, 2004), NAA (0.5 mg l⁻¹) and N6-(2-isopentenyl)-adenine (2.0 mg l⁻¹) for pseudobulb multiplication in Otochilus alba (Mukhopadhyay and Roy, 1994), IBA (4.14 μM) for higher number of shoots per PLB in Encyclia mariae; BAP (22.21 μM) and NAA (5.37 μM) for PLB derived shoot elongation in Encyclia mariae (Díaz and Álvarez, 2009). Sheelavantmath et al. (2005) used cytokinins BAP, KN, TDZ (0.5, 1.0, 2.0, 5.0 μM), auxins NAA and IAA (0.5, 1.0, 2.0, 5.0 μM) as well as CW (5, 10, 15%) for PLB induction in Aerides crispum protocorms and found BAP to be the best for the purpose in 5-8 weeks. The size of protocorms of Grammatophyllum speciosum increased in BAP and 2,4-D containing medium. However, fresh mass was decreased and root development delay at the higher concentrations of BAP (Monti et al., 2008). Basal MS medium was put to utility for PLB proliferation in D. huoshanenense (Luo et al., 2003). Lin et al. (2000) reported calli formation in seed derived protocorms of Paphiopedilum hybrid when grown on half strength MS medium fortified with 2,4-D (1.0-10.0 mg l⁻¹) and TDZ (0.1-1.0 mg l⁻¹); these calli proliferated more on half strength MS medium containing 2,4-D (5.0 mg l⁻¹) and TDZ (1.0 mg l⁻¹). The improvement of shoot growth rate and shoot length of Dactylorhiza species was enhanced by cytokinins N⁶-(2-isopentenyl) adenine or BAP and their combination with auxin (IBA) (Wotavová-Novotná et al., 2007). The protocorms of Encyclia mariae induced callus formation with high regenerative potential in the form of PLBs that eventually differentiated into shoots on media containing NAA, IBA, or IAA (Díaz and Álvarez, 2009).

In the present taxon, the effect of sucrose was assessed for seedling development as well as PLB and pseudobulb proliferation; 2% was most fruitfully utilized for the purpose. The higher concentration of 2% also resulted in higher dry weight irrespective of the medium or organic additives. 2% sucrose was similarly utilized by Martin and
Madassery (2003) (*Ipsea malabarica*) and both 2 and 3% sucrose was used in *Cymbidium* hybrid (Nagaraju and Upadhayaya, 2001). However, 3% sucrose was shown to be effective for seedling formation in *Coelogyne punctulata* and *Cymbidium elegans* and sucrose, D-fructose, and D-glucose were preferred (Sharma and Tandon, 1990). Higher concentration of 6 or 8% of sucrose was reported to lead to a hastened bulb development in *Ipsea malabarica* (Martin and Madassery, 2003). In the current study, medium without sucrose did not lead to positive results, nevertheless, Sinha *et al.* (2001) effectively used medium without any sucrose for PLB proliferation in *Renandes*. The seedling development of *Dactylorhiza* species was stimulated with the application of glucose and sucrose at concentration of 10 g dm⁻³ each (Wotavová-Novotná *et al.*, 2007).

During the study at hand, both M and MS media were fruitfully used for root induction in *Malaxis acuminata*. The utility of MS medium was indicated by Díaz and Álvarez (2009) (*Encyclia mariae*), Mahendran and Narmatha Bai (2009) (*Satyrium nepalense*), Martin and Madassery (2006) (*Ipsea malabarica*), Martin *et al.* (2005) (*Dendrobium* hybrid), Mukhopadhyay and Roy (1994) (*Otochilus alba*), Nagaraju and Upadhayaya (2001) (*Cymbidium* hybrid), Nayak *et al.* (2002) (*D. aphyllum* and *D. moschatum*; *Cymbidium aloifolium* and *D. nobile*), and Seen and Latha (2000) (*Vanda coerulea*). However, half strength MS medium was used for the purpose by Martin and Madassery (2006), Martin *et al.* (2005), and Nagaraju and Upadhayaya (2001). Half strength M and Phytamax media were used for root induction in *Epidendrum ibaguense* (Hossain, 2008). KC and VW media were used for root initiation in *Dendrobium fimbriatum* (Roy and Banerjee, 2003) and *V. coerulea* (Malabadi *et al.*, 2004), respectively.

Presently, the induction of rooting was possible on MS as well as M media supplemented with BH (60 g l⁻¹) or CW (10%). On a similar note, rooting of shoots of *V. coerulea* [banana pulp (35 g l⁻¹) and NAA (1.08 μM) (Seeni and Latha, 2000)] and *V. spathulata* [banana pulp (75 g l⁻¹) and IAA (5.7 μM) (Decruse *et al.*, 2003 a)] occurred on medium enriched with BH along with auxins. Root formation was induced in growth regulator-free medium in *Cymbidium* hybrid (Nagaraju and Upadhayaya, 2001), *Dendrobium fimbriatum* (Roy and Banerjee, 2003) and *Dendrobium* hybrid (Martin *et al.*, 2005), or in the presence of IBA [(C. aloifolium and *D. nobile*; Nayak *et al.*, 2002; 9.8
Discussion

(iM); (Satyrium nepalense, Mahendran and Narmatha Bai, 2009; 14.76 μM); (C. aloifolium, D. aphyllum and D. moschatum; Nayak et al., 2002 (10.8 μM)), IAA [Epidendrum ibaguense, Hossain, 2008, 0.5-1.0 mg/l), (Vanda coerulea, Malabadi et al., 2004, 11.42 μM]), IAA or IBA [Encyclia mariae (5.71 μM and 4.14 μM, respectively) (Diaz and Alvarez, 2009)], and TRIA (triacontanol) [D. nobile; Malabadi et al., 2005 (2.0 μg l⁻¹)]. The root growth rate and root length of seedlings increased in the presence of IBA and NAA (Wotavová-Novotná et al., 2007). At low concentrations of 2.4-D, roots developed well in Grammatophyllum speciosum while browning occurred at higher concentrations (Montri et al., 2008). In the present study, only agar gelled M and MS media were utilized for root formation. However, Diaz and Álvarez (2009) reported 20% higher rooting in liquid media than in agar-gelled medium in Encyclia mariae.

In the present study, root formation occurred within 4-6 weeks after the PLBs or shoots were transferred to BH containing medium. Likewise, root formation was observed within 3-9 weeks in Vanda spathulata (Decruse et al., 2003a) and 3-4 weeks in V. coerulea (Seeni and Latha, 2000).

5.1.2.3 Lab to land transfer of in vitro plantlets

Micropropagated plantlets are typically grown under an environment that provides minimum stress and optimal growth conditions. The in vitro conditions include an artificial nutrient medium, low light regimes, and high relative humidity which contribute to a phenotype with abnormal morphology, anatomy, and physiology (Kozai, 1991; Preece and Sutter, 1991). The most striking features being retardation in the development of cuticle, epicuticular waxes, functional stomatal apparatus, and conducting tissues (Diettrich et al., 1992; Santamaria et al., 1993; Sutter, 1988). The in vitro raised plantlets are photomixotrophic and bear leaves with low chlorophyll content and photosynthetic rates which impede growth (Grout and Millan, 1985). In vitro plants possess thin, soft, photosynthetically hypoactive leaves (Gangopadhyay et al., 2002) which makes them vulnerable. Acclimatization, which is the transition from in vitro to ex vitro conditions, is critical as the above mentioned abnormalities must be corrected to ensure survival and continued normal plant growth (Preece and Sutter, 1991).
In the present taxon, the rooted plants were removed from the flasks and washed free of agar in running tap water and transplanted in plastic or clay pots. In a similar way, plantlets were washed prior to transplanting by Bejoy et al. (2004) (*Peristeria elata*), Nagaraju et al. (2002) (*Cymbidium* hybrid), Nayak et al. (2002) (*C. aloifolium* and *Dendrobium nobile*), Nongdam et al. (2006) (*C. lowianum*), Pyati et al. (2002) (*D. macrostachyum*), Seeni and Latha (1990) (*Phalaenopsis*), and Singh et al. (2006 b) (*Aerides odorata, Dactylorhiza pierardii, Phaius tankervilliae, and Rhynchostylis retusa*). During the current study, the plantlets were then either treated with fungicide (Mancozic powder, 0.5%) for 5 min before transferring them to earthen pots or were transferred directly and success was achieved in both the cases. Fungicide treatment was also given by Bejoy et al. (2004) (1 g l⁻¹ Indofil-M45, 5 min), Nagaraju et al. (2002), Nongdam et al. (2006) (0.01%, 15-20 min), Seeni and Latha (1990) (0.1% Dithane M45 solution). During the investigations at hand, potting mixture was not provided with any fungicide treatment though it was washed with distilled water twice for leaching out the salts. Seeni and Latha (1990) stated that the fungicide treatment of the pots and potting mixture was unnecessary. The pots were covered with plastic covers initially, for a period of approx. one month or till the plantlets formed new leaves and roots to avoid desiccation. However, Seeni and Latha (1990) did not observe any signs of withering of *Phalaenopsis* plants when not covered with plastic covers. The plantlets were initially kept in a shaded cool room before shifting them to high humidity glass house in the present study.

The plantlets were either directly shifted to pots or transferred after hardening procedure; 40% plantlets survived in the latter case. Nonetheless, Seeni and Latha (1990) reported that acclimatization was not a pre-requisite for survival of the transferred plantlets of *Phalaenopsis*; Seeni and Latha (2000) obtained success in *Vanda coerulea* without hardening. Decruse et al. (2003 b) and Nayak et al. (2002) also transferred the plantlets of *Vanda spathulata, Cymbidium aloifolium* and *Dendrobium nobile*, respectively without prior hardening. However, Singh et al. (2006 b) subjected the plantlets of *Aerides odorata, Dactylorhiza pierardii, Phaius tankervilliae, and Rhynchostylis retusa* to liquid medium containing 1% sucrose on cotton wads for three weeks before shifting them to the net house. Anirudhan and Nair (2006) used moist coir
Discussion
to harden Vanda Poepoe Diana plantlets for 2 weeks. Nagaraju et al. (2002) reduced the salt concentration for hardening the plants of Cymbidium hybrid whereas Nongdam et al. (2006) did not provide any growth regulators in the medium for a few weeks before the transfer of C. lowiamum. Sheelavantmath et al. (2000) provided half strength MS medium to the plantlets of Geodorum densiflorum before shifting them to the pots. Bhadra and Hossain (2004) also followed the acclimatization procedure in Micropera pallida. In the current investigations, full strength MS liquid medium (without sucrose and vitamins) was sprayed on the potting mixture before the transfer and the plantlets were also sprayed with it every week; the plants were watered with distilled water till over a month. Likewise, Seeni and Latha (2000) continued treating the transferred Vanda coerulea plantlets with nutrients till 6 months. The transferred plantlets were incubated in shade areas with 25±2°C temperature regime and a light of 35 μmol m⁻² s⁻¹ intensity before transferring them to the greenhouse. Similar observations were made by Nayak et al. (2002) in Cymbidium aloifolium and Dendrobium nobile. Seedlings of Calopogon tuberosus readily acclimatized to greenhouse conditions (Kauth et al., 2006).

In the current study, the potting mixture consisted of autoclaved river sand: peat moss: silica in a ratio of 1:1:1 with appropriate success. Various combinations of potting mixtures have been utilized productively by earlier workers [(sterilized coarse sand: vermiculite, Aerides odorata, Dactylorhiza pierardii, Phaius tankervilliae, and Rhynchosystis retusa, Singh et al., 2006 b), (coconut husk growing medium, Cyrtopodium punctatum, Dutra et al., 2009), (peatmoss: wood charcoal: bricks, 1:1:1, Cymbidium aloifolium and Dendrobium nobile, Nayak et al., 2002), (white moss: leaf mould: Farm Yard manure, 1:1:1, Cymbidium hybrid, Nagaraju et al., 2002), (brick pieces: pine bark/charcoal pieces: moss, 1:1:1, C. lowiamum, Nongdam et al., 2006), (sand: soil: brick pieces: charcoal pieces, 1:1:4:4, D. macrostachyum, Pyati et al., 2002), (coconut husk-ship, Grammatophyllum speciosum, Montri et al., 2008), (moist coir: saw dust: coal, 1:1:1, Micropera pallida, Bhadra and Hossain, 2004), (broken sand: charcoal: river sand, 1:1:1, Peristeria elata, Bejoy et al., 2004), (broken tiles, Phalaenopsis, Seeni and Latha, 1990), (peatmoss: perlite, 1:1, Phalaenopsis, Young et al., 2000), (vermicompost containing leaf litter: cow dung in a ratio of 1:1 along with sand: coconut husk, 1:1:1, Satyrium nepalense, Mahendran and Narmatha Bai, 2009), (charcoal chips: broken tiles,
Discussion

2:1, *Vanda coerulea*, Seeni and Latha, 2000), (coconut husk: charcoal, 1:1, *Vanda Poepoe Diana*, Anirudhan and Nair, 2006), and (charcoal chips: coconut husk: broken tiles, 2:2:1, *V. coerulea*, Malabadi et al., 2004; *D. nobile*, Malabadi et al., 2005)]. Kishor et al. (2006) reported that brick pieces: charcoal: *Sphagnum* moss (2:1:1) was more fruitfully utilized than brick pieces: charcoal: tree fern (2:1:1) in *V. coerulea* x *Ascocentrum auranticum* survival. Seeni and Latha (1990) arranged the different potting mixture ingredients in a descending order of their efficiency i.e. broken tiles, charcoal chips, dried Cassava stem cuttings, outer shells of rubber seeds, and coconut husk. *Bletia purpurea* seedlings acclimatized in Fafard Mix 4 potting medium developed significantly longer roots; corm formation occurred regardless of the potting media used (Dutra et al., 2008).

Survival rate of the present species was 40%. However, a higher survival rate has been reported in *Aerides odorata*, *Dactylorhiza pierardii*, *Phaius tankervilliae*, and *Rhynchostylis retusa* (Singh et al., 2006 b; 100%), *Cattleya maxima* and *Cymbidium* hybrid (Nagaraju et al., 2003), *Cyrtopodium punctatum* (Dutra et al., 2009, 90%), *Dendrobium nobile* (Malabadi et al., 2005; 92%), *Epidendrum ibaguense* (Hossain, 2008, 90%), *Micropera pallida* (Bhadra and Hossain, 2004; 77%), *Peristeria elata* (Bejoy et al., 2004; 96%), *Phalaenopsis* (Seeni and Latha, 1990; 100%), and *Vanda coerulea* [(Malabadi et al., 2004, 98%), (Seeni and Latha, 2000; 95%), and (Sheelavanmath et al., 2000; 95-100%)]. Solarova and Pospisilova (1997) and Vyas and Purohit (2003) suggested approaches to enhance the survival of transferred *in vitro* plants; photoautotrophic multiplication of shoots on a medium without sucrose but with high CO$_2$ followed by their hardening and acclimatization.

5.2 HAIRY ROOT FORMATION

5.2.1 *Solanum xanthocarpum*

5.2.1.1 Explants used

In the present study, leaf segments and callus pieces of *Solanum xanthocarpum* were used as explants for infection with *Agrobacterium rhizogenes* A4 strain. The leaf segments responded positively and 13.24% and 16.11% leaf explants formed hairy roots in the absence and presence of acetosyringone treatment, respectively. The results
Discussion

indicated that when the leaves were placed on the abaxial side, the hairy root formation response was higher than when they were placed on the adaxial side. Similarly, even the untreated leaf explants formed higher number of control roots when placed on the abaxial side (5.10 and 9.96% control roots in un-treated leaves placed on the adaxial and abaxial ends, respectively). Infection of calli with \textit{A. rhizogenes} did not lead to any hairy root formation. However, success was achieved when calli of \textit{S. laciniatum} were co-cultured with \textit{A. rhizogenes} strains (Sadykova \textit{et al.}, 1991).

5.2.1.2 Adaxial vs abaxial side of the explant

Presently, it was observed that when the leaves were placed on the adaxial side (dorsal side touching the medium), the number of control or hairy roots formed was more than when they were placed on the abaxial side. This indicated a polarity in root formation. Ottaviani \textit{et al.} (1990) reported more root formation at the basal side of the leaf segments in \textit{S. tuberosum}. This could be due to polarity in the stem and to a higher endogenous auxin concentration in cells nearer to the base of the stem. Polar basipetal transport of native auxins is a well known physiological process in stems of higher plants. Auxin is, in addition to the presence of T-DNA, required for induction of hairy roots in carrot (\textit{Daucus carota}) discs. Inoculation of decapitated hypocotyls with an intact root system gave rise to direct shoot formation from the site of inoculation (Damgaard and Rasmussen, 1991) and it was demonstrated that location of site of infection in the hypocotyls affected the ability of hairy root formation in \textit{Brassica napus}. Inoculation at the basal part of hypocotyls resulted in a higher yield of adventitious roots compared to inoculation at the apical part. Dobigny \textit{et al.} (1996) inoculated the stem fragments of cultivars of \textit{Solanum tuberosum} as explants. The stem fragments were placed in both the positions, the lower and upper end being in contact with the medium. The hypocotyl segments of \textit{Lycopersicon esculentum} were used so that their basal sides touched on freshly prepared bacteria cultures; later, the inoculated segments were plunged, basal end up, into solidified MS medium (Peres \textit{et al.}, 2001).
5.2.1.3 Explant size

The optimum size of the leaf explants as well as the response of injury on the different sides of the leaf explants was also studied in the present study. It was found that the leaves of 0.5x0.5 cm dimensions, cut on all the sides, and the 0.3x0.5 cm sized leaf with serrated margin, also cut from all the sides, were the best suited for higher hairy root or control root formation. Wounding enhanced the transformation frequency in the current investigations. Wounding is the most effective biological trigger for shifting cells potentially competent for regeneration to the competent state. Apart from stimulating dedifferentiation, wounding also led to the excretion of chemical signal that induces Agrobacterium infection (cf. Kuta and Tripathi, 2005). Similar to our studies, Jaziri et al. (1994) used leaf segments (ca. 0.5x0.5 cm) of Atropa belladonna as explants for transformation with Agrobacterium rhizogenes. Young leaves from glasshouse grown plants of Allium cepa, Antirrhinum majus, Brassica campestris, Glycine max, Nicotiana tabacum were cut into pieces approximately 8x8 mm (Godwin et al., 1991). Stem fragments (5 mm long) of in vitro grown cultivars of S. tuberosum were used as explants for treatment with A. rhizogenes (Dobigny et al., 1996).

5.2.1.4 Number of days taken for initiation of response

During the current investigations, earliest hairy root response was obtained in 5-18 days (an average of 9.94 days) in Solanum xanthocarpum; the un-treated leaf explants showed the initiation of control roots in 6-10 days (an average of 9.48 days). Earliest response was observed within one week in the case of hairy roots of Atropa, Datura, Hyoscyamus, and Scopolia induced by the infection of the leaf explants with A. rhizogenes which was similar to our study (Knopp et al., 1988). Hairy root formation occurred after 14-20 days in Atropa belladonna (Sharp and Doran, 1990), and Lycopersicon esculentum (Peres et al., 2001); 20 days in Hyoscyamus officinalis (Murakami et al., 1998); two weeks in Nicotiana rustica and Beta vulgaris (Hamill et al., 1986), and Datura stramonium (Maldonado-Mendoza et al., 1993); two to four weeks in S. tuberosum (Ooms et al., 1985); and two to five weeks in A. belladonna (Kamada et al., 1986).
5.2.1.5 Strain used

In the present study, *Agrobacterium rhizogenes* A4 strain was utilized for the infection of explants. Similarly, A4 strain was used to initiate hairy roots in *Solanum aviculare* (Subroto and Doran, 1994), 24 different Solanaceae species using A4 as well as 8196 strain (Knopp et al., 1988), *Atropa belladonna* (Kanokwaree and Doran, 1997; Kwok and Doran, 1995; Subroto et al., 1996; Williams and Doran, 2000; Yu and Doran, 1994), *Nicotiana tabacum*, *Datura metel*, Duboisia hybrid, (Moyano et al., 1999), *S. dulcamara* (McInnes et al., 1991), *N. tabacum* and *S. tuberosum* (Ondrej et al., 1989; Schmulling et al., 1993), horse radish (*Armoracia lapathifolia*) (Noda et al., 1987), and *Panax ginseng* (Mallol et al., 2001).

5.2.1.6 Acetosyringone

An average of 13.24% and 16.11% leaf explants formed hairy roots in the absence and presence of acetosyringone (100μM/ml, a natural wound response molecule) treatment, respectively. Acetosyringone also led to a higher percent response when the response of leaves placed abaxially or adaxially was compared to that in the absence of acetosyringone treatment. Acetosyringone also proved beneficial in promoting an earlier response when the leaf explants were treated with A4+acetosyringone (9.94 days); while in the absence of acetosyringone hairy roots were formed in 11.1 days. The un-treated leaf explants showed the initiation of control roots in 9.48 days. Acetosyringone has been known to enhance transformation efficiency due to activation of vir genes in *A. tumefaciens* (Kumar et al., 2006). Hence, we assume that the enhancement in transformation by acetosyringone treatment may be due to the activation of vir genes which is absolutely required for the T-DNA delivery to plant tissues.

In the present study, *Agrobacterium* culture was not given any prior acetosyringone treatment, still we got up to 13.24 and 16.11% transformation in the leaf explants untreated and treated with acetosyringone, respectively. Sheikholeslam and Weeks (1987) enhanced the transformation frequency in *Arabidopsis thaliana* by giving a prior treatment of acetosyringone to *Agrobacterium* culture; the rate of transformation ranged from 55 to 63% when acetosyringone (20 μM) was added to an *A. tumefaciens* culture 16 h prior to incubation with leaf segments; the transformation rate being approx.
2-3% in the absence of acetosyringone. Sriskandarajah and Goodwin (1998) added 100 
μM acetosyringone and 10 μM glucose to the bacterial suspension to enhance the 
transformation frequency in apple leaf explants. 150 μM of acetosyringone was added to 
shoot induction medium for co-cultivation of almond leaves with Agrobacterium, in 
addition to TDZ, IAA, and 2, 4-D in MS medium and co-cultivated for three days. It was 
found that shoots regenerated from explants subjected to 150 μM acetosyringone were 
more vigorous and healthier under selection pressure and the additional presence of 
acetosyringone in the induction medium led to an additional 100-fold increase in 
transformation efficiency (Costa et al., 2006). Kumar et al. (2006) reported that infection 
of leaf explants of N. tabacum by manual wounding resulted in induction of hairy roots 
originating from the mid vein; however, acetosyringone treatment (50, 100, 150 μM) 
resulted in induction of hairy roots from all over the surface of the leaf explants. 
However, contrary to our results, the evidence given by Godwin et al. (1991) suggested 
that acetosyringone (200 μM) may repress virulence in some strain/plant species 
interactions when explants of five plant species (Allium cepa, Antirrhinum majus, 
Brassica campestris, Glycine max, and Nicotiana tabacum) were co-cultivated with 
Agrobacterium tumefaciens strains.

5.2.1.7 Co-cultivation time

During the current investigations, the explants were suspended in the bacterial 
suspension in an autoclaved jam bottle for 10 min for Solanum xanthocarpum and 15 min 
for Malaxis acuminata. The explants of both the species were later on transferred to the 
co-cultivation medium and kept in the same for two days. Jaziri et al. (1994) co- 
cultivated the explants of Atropa belladonna in the bacterial suspension for one day. 
However, there are some reports where the co-cultivation time was increased. 
Maldonado-Mendoza et al. (1993) co-cultivated Datura stramonium explants for two 
weeks whereas Jaziri et al. (1988) incubated the hypocotyl explants of D. sramonium and 
Hyoscyamus niger with A. rhizogenes for three to four weeks.

5.2.1.8 Medium composition

In the present taxon, full strength hormone-free MS medium containing 
acetosyringone (100 μM/ml) was used in the case where the explants were treated with
A4+acetosyringone (100 µM/ml) for two days; while no acetosyringone was added to the medium in the case where the explants were not treated with acetosyringone. Hormone-free medium was similarly utilized by Knopp et al. (1988) in Atropa, Datura, Hyoscyamus, and Scopolia species, Komaraiah et al. (2003) in Solanum tuberosum, Shimomura et al. (1991) in D. innoxia, H. albus, H. niger, and Scopolia tanguica, Spano et al. (1988) in Nicotiana tabacum, Zehra et al. (1988) in H. albus and H. muticus. In the current study, no prior treatment was given to the explants before infection with the bacterium. However, NAA pre-treatment was given to the explants of Solanum tuberosum (Dobigny et al., 1995, 1996; Vardja et al., 2000). NAA was added to the medium containing antibiotic when the explants were incubated post-infection to enhance the transformation efficacy in Lycopersicon esculentum and S. tuberosum (Arican et al., 1997). Devi and Rani (2002) advocated the use of IBA enriched MS medium for the formation of extensively branched hairy roots in Helianthus annuus x H. tuberosus.

Presently, sucrose (30 g l\(^{-1}\)) sucrose was added to the co-cultivation medium as well as the liquid MS medium for hairy root culture. In accord with the current study, 3% sucrose was utilized by Komaraiah et al. (2003) in N. tabacum and Shimomura et al. (1991) in D. innoxia, H. albus, H. niger, and Scopolia tanguica cultures. However, the highest values for fresh weight of hairy roots of Centaurea calciapa were attained between 30 and 50 mg l\(^{-1}\) of sucrose (Lourenco et al., 2002). It was found that optimal sucrose concentration varied from 2, 5 g l\(^{-1}\) to 25 g l\(^{-1}\) depending on the potato cultivar (Vardja et al., 2000).

5.2.1.9 Antibiotic treatment

After co-cultivation, the explants were shifted to hormone-free MS medium containing Augmentin (300 mg l\(^{-1}\)) as an agent to kill the agrobacterium. Augmentin treatment was repeated for three sub-cultures after 15 days or till the bacterium growth persisted. Augmentin has also been, similarly, reported to suppress the growth of Agrobacterium tumefaceins in Lycopersicon esculentum cultures at a concentration of 300 mg l\(^{-1}\) (Ieamkhang and Chatchawankanphanich, 2005). Sharp and Doran (1990) and Subroto and Doran (1994) and used cefotaxime to clear the roots of bacteria in hairy roots of Atropa belladonna and S. aviculare, respectively. Kittipongpatana et al. (1998)
used 0.6 to 1.0 g l\(^{-1}\) of ampicillin to eliminate excess bacteria. Parr and Hamill (1987) also added ampicillin to hairy roots of *Nicotiana* species but at a lower concentration (0.5 g l\(^{-1}\)) to remove residual *Agrobacterium*. Shimomura *et al.* (1991) and Sauerwein and Shimomura (1991) utilized Claforan (0.5 g l\(^{-1}\)) for the purpose. Zehra *et al.* (1988) utilized cephalaxin (1 g l\(^{-1}\)) in *H. albus* and *H. maticus* cultures.

In the present study, no antibiotic was added to the liquid medium once the hairy roots were transferred to liquid MS medium. However, Parr and Hamill (1987) added 0.25 g l\(^{-1}\) of ampicillin to the liquid culture of hairy roots of *Nicotiana* during each subculture. The transformed roots of *S. tuberosum* were transferred to solid MS medium with 200 mg l\(^{-1}\) carbenicillin to eliminate *A. rhizogenes*. Hairy roots, free form the bacterium, were transferred to the above medium with agar (Komaraih *et al.*, 2003). In the present study, the roots were excised and transferred to liquid medium devoid of any antibiotic. However, the well growing, branching roots of *Atropa*, *Datura*, *Hyoscyamus*, and *Scopolia* species were excised and grown on medium containing 100 mg l\(^{-1}\) Cefotaxime (500 mg l\(^{-1}\) carbenicillin in the case of *H. maticus*).

### 5.2.1.10 Medium composition for liquid hairy root culture

In the current study, 10 root tips (approx. 1.0 cm in length) were transferred to 100 ml liquid MS medium. Jaziri *et al.* (1988) excised terminal pieces (approx. 20 mm long) from emerging hairy roots of *Datura stramonium* and *H. niger*. Two pieces of transformed root of *D. stramonium* approx. 7 cm long were utilized for further growth (Hilton and Rhodes, 1994). Komaraih *et al.* (2003) used 3-6 cm long root tips of *S. tuberosum*.

After the emergence of hairy roots, the tips were excised and transferred to liquid growth regulator-free full strength MS medium supplemented with 30 g l\(^{-1}\) of sucrose. Similarly, liquid MS medium without growth regulators was utilized for the growth of hairy roots by Macek *et al.* (1994) (*S. nigrum*), Moyano *et al.* (1999) (*N. tabacum*), Shimomura *et al.* (1991) (*D. innoxia*, *H. albus*, *H. niger*, and *Scoparia tangutica*), and Yu *et al.* (1996) (*S. aviculare*). Liquid B\(_5\) medium was used for hairy root growth in *D. stramonium* (Baiza *et al.*, 1998) and *Solanum aculeatissimum* (Ikenaga *et al.*, 1995). However, solid half strength B\(_5\) medium was used in *Datura metel* and *Duboisia* hairy
roots (Moyano et al., 1999). Solid medium was also utilized for hairy root growth of *Atropa, Datura, Hyoscyamus,* and *Scopolia* (Knopp et al., 1988). Either solid or liquid medium was used for the hairy root culture maintenance of *Solanum mauritianum*; reduced root growth was observed when the medium strength was decreased to half (Drewes and van Staden, 1995). However, the hairy root growth of *S. aculeatissimum* was shown to enhance when auxin (100 µg l\(^{-1}\)) was added to the medium; the addition of 2,4-D inhibited growth (Ikenaga et al., 1995). In contrast to the present study, transformed root segments of *A. belladonna* were cultured onto half strength MS solid medium supplemented with NAA (0.5 µM) and BAP (5.0 µM) (Jaziri et al., 1994).

Similar to the present study, sucrose was added at 3% concentration for hairy root growth and maintenance in *Atropa belladonna* (Kanokwaree and Doran, 1997), *Datura stramonum* (Baiza et al., 1998), *Nicotiana tabacum* (Moyano et al., 1999), and *Solanum aculeatissimum* (Ikenaga et al., 1995). However, decreased root growth rates of *S. mauritianum* hairy roots were recorded when 3% glucose was substituted by 3% sucrose (Drewes and van Staden, 1995). In addition to 3% sucrose, myo-inositol (0.1 g l\(^{-1}\)) was added to the culture medium (Drewes and van Staden, 1995). Yu et al. (1996) utilized either 3% or 6% sucrose for maintaining *S. aviculare* hairy roots. In antagonism to the current study, Jaziri et al. (1994) utilized a lower concentration of 1.5% sucrose for hairy root culture of *A. belladonna* whereas Sauerwein and Shimomura (1991) utilized a higher concentration of 5% in *Hyoscymus albus.*

Presently, the liquid hairy root cultures were maintained in an orbital shaker with 80-120 rpm to provide aeration and in dim light at 25°C. Hairy roots of *S. khasianum* were cultivated for 8 weeks at 100 rpm, 25°C, 35 µmol m\(^{-2}\) s\(^{-1}\) of light with a 14 h photoperiod (Jacob and Malpathak, 2005 a). Similarly, Jacob and Malpathak (2005 a) and Parr and Hamill (1987) maintained the hairy root cultures of *S. khasianum* under the above culture conditions. Boitel-Conti et al. (1996, 2000) also followed the above regime in hairy root cultures of *D. innoxia.* However, Shimomura et al. (1991) maintained the hairy root cultures of *H. albus* and *H. niger* under dark conditions. However, 3,000 lux continuous illumination regime was followed in *S. aviculare* roots (Yu et al., 1996).

During the current study, the roots emerged from the wound sites of leaf explants (after one-two weeks) were transferred to liquid MS hormone-free medium without any
antibiotic added to this liquid medium. However, *S. tuberosum* roots were excised and placed on medium supplemented with 200 mg/l carbenicillin to eliminate *A. rhizogenes* (Komaraiah et al., 2003). Similar to the present study, Sauerwein and Shimomura (1991) eliminated the bacteria from the infected explants of *H. albus* on MS solid medium, though the antibiotic used was different (Claforan, 0.5 g/l).

Some browning of the root tips was observed in the current investigations which could be due to the reason that solasodine in the medium was toxic to the transformed roots and caused browning of the root tips. Similar browning of the root tips was observed in *S. khasianum* (Jacob and Malpathak, 2005a). After 5-6 weeks of culture, if left without a subculture, the hairy roots turned brown in the course of the present investigations. Similarly, browning of hairy roots of *Coleus forskohlii* was observed after 5 weeks in MS and B5 media (Li et al., 2005). In the present study, regeneration of a shoot from a few roots was observed in the hormone-free liquid MS medium. Hairy root cultures of *S. khasianum* showed regeneration of 50-70 mm long shoots after treatment with IAA and KN (Jacob and Malpathak, 2005a). Similarly, shoot outgrowth from hairy roots of potato cv. Bintje occurred on liquid MS medium in the absence of hormones (Ottaviani et al., 1990).

### 5.2.1.11 Percentage of transformation

The leaf segments responded positively and 13.24% and 16.11% leaf explants formed hairy roots in the absence and presence of acetosyringone treatment, respectively. The leaf explants were placed abaxially or adaxially and it was found that in the absence of acetosyringone treatment, 9.17% and 11.86% explants responded with hairy roots when placed on the adaxial and abaxial sides, respectively; while the percentage of response when the explants were treated with acetosyringone along with A4 strain was 11.9% and 17.02%, respectively. In the present study, 17% *S. xanthocarpum* leaf explants responded with the initiation of hairy roots. However, as many as 80% of the explants of *Atropa, Datura, Hyoscyamus,* and *Scoporia* species infected with *Agrobacterium rhizogenes* strain A4 were found to have the initiation of hairy roots (Knopp et al., 1988). Callus explants were unable to produce any hairy roots while $3.5 \times 10^3$ calli of *S. nigrum* were transformed; the difference could be the strain used (*A. rhizogenes* harbouring...
agropine-type Ri plasmid, pRl5834) (Wei et al., 1986). The frequency of hairy roots ranged from 17% to 92% when Han et al. (1997) tried to inoculate species of *Populus* with wild-type *A. rhizogenes*. The transformation efficiency of conditioned shoot explants of apple (conditioning in liquid MS medium for 4 days prior to co-cultivation) varied from 1.2 to 2.6% (Sriskandarajah and Goodwin, 1998).

Application of antioxidants, addition of acetosyringone and optimization of pre-culture conditions suppress the *Agrobacterium*-induced hypersensitive necrotic response in target plant tissues, thereby enhancing stable transformation. Pre-culture of explants prior to *Agrobacterium* infection on media containing auxin with or without cytokinins was good method of inducing cells to undergo dedifferentiation and may serve as rejuvenating treatment to the explant. Juvenile plant cells may be more susceptible to *Agrobacterium* infection than differentiated old cells (Kuta and Tripathi, 2005).

5.2.1.12 Growth of Control Roots

In the present study, the control leaf explants formed normal tap roots. The percentage of control explants forming roots was 11.13%. In contrast, the controls failed to initiate roots on intact seedlings or cultured segments in presence or absence of IBA in *Pinus radiata* while the treated ones (*A. rhizogenes* or IBA) formed roots (Leung and Li, 2003). Rooting could also not be induced in control shoots in *Helianthus annuus x H. tuberosus* on MS basal medium, only sparse and unbranched roots were produced on MS medium supplemented with IBA (Devi and Rani, 2002). Control roots were grown in basal MS medium containing 3% sucrose without the addition of any exogenous plant growth regulator in the current investigation. However, Baiza et al. (1998) grew normal root culture of *Datura stramonium* in B5 medium with IBA (0.1µM) and 3% sucrose.

5.2.2 Malaxis acuminata

5.2.2.1 Explants used

In the present set of investigations, protocorms, PLBs, and leaf sections (0.5-1.0 cm in length) were used as explants for infection with *Agrobacterium rhizogenes*. PLBs are ideal for utilization as explants for transformation experiments as they contain a higher concentration of coniferyl alcohol than other tissues (Yu et al., 2001). Similarly,
PLBs were used as explants for the transformation of various orchid species with *A. tumefaciens*, namely *Dendrobium* Sonia transformation (Pirla et al., 2005), *D. nobile* (Men et al., 2003), *Dendrobium* Madame Thong-In (Yu et al., 2001), *Phalaenopsis* species and *Zygopetalum mackayii* (Yoshiyuki et al., 2001, 2002). PLBs derived from protocorms of *Oncidium* orchid were the target explants for transformation (Liau et al., 2003). Immature protocorms were used for transformation of *Dendrobium* Sonia (Pirla et al., 2005) and *Phalaenopsis* orchid (Mishiba et al., 2005). Earlier workers have utilized various explants for transformation with *A. tumefaciens*. Embryogenic callus of *Neofinetia falcata*, rhizome of *Cymbidium niveo-marginatum*, and shoot primordia of *Schomburgkia sanderiana* were used as explants for transformation with *A. tumefaciens* (Yoshiyuki et al., 2001, 2002). Cell clumps derived from friable calli from flower stalk cuttings of *Phalaenopsis* were used for transformation experiments (Belarmino and Mii, 2000).

5.2.2.2 Strain used

*Agrobacterium rhizogenes* strain A4 was used for the present set of experiments. However, there have been no reports of an orchid transformation with *A. rhizogenes*. Nevertheless, transformations with *A. tumefaciens* have been reported; *Dendrobium* Sonia (Pirla et al., 2005); *D. nobile* (Men et al., 2003); *Dendrobium* Madame Thong-In (Yu et al., 2001); *Oncidium* orchid (Liau et al., 2003); *Neophytina falcata*, *Cymbidium niveo-marginatum*, *Schomburgkia sanderiana*, and *Zygopetalum mackayii* (Yoshiyuki et al., 2001; Yoshiyuki et al., 2002); *Phalaenopsis* orchid (Belarmino and Mii, 2000); and *Phalaenopsis* (Mishiba et al., 2005).

5.2.2.3 Method of infection

The explants were not subjected to a pre-culture treatment prior to infection with the bacteria in the current study. The *in vitro* leaves, protocorms, and PLBs of *Malaxis acuminata*, growing in basal MS medium, were cut along all sides and suspended in the bacterial suspension for 15 min, with intermittent shaking. The explants were then inoculated on full strength growth regulator-free MS medium. However, in contrast to the current study, *Dendrobium nobile* PLBs were pre-cultured on MS medium containing...
Discussion

BAP (1.0 mg l\(^{-1}\)) and acetosyringone (100 \(\mu\)M) prior to incubation with \textit{A. tumefaciens} for two days (Men \textit{et al.}, 2003). Prior sub-culture of \textit{Phalaenopsis} protocorms was carried out in acetosyringone containing medium 2 days before bacterial inoculation (Mishiba \textit{et al.}, 2005).

5.2.2.4 Infection time

In the present study, the explants were subjected to bacterial suspension for only 15 min followed by their subsequent presence in the antibiotic free medium for two days (in the absence or presence of acetosyringone; 100 \(\mu\)M). In consonance with current investigations, Pirla \textit{et al.} (2005) inoculated the PLBs and protocorms of \textit{Dendrobium} Sonia with \textit{Agrobacterium tumefaciens} for 15 min. In contrast to the present study, Men \textit{et al.} (2003) immersed \textit{D. nobile} explants in bacterial suspension for 30, 45 or 60 min; found that 30 min inoculation period gave the highest transformation efficiency, compared to that of 45 or 60 min; the transformation efficiency reducing to 3-4% from 18% on increasing the incubation period from 30 to 60 min. Liau \textit{et al.} (2003) and Yu \textit{et al.} (2001) immersed the explants of \textit{Oncidium} and \textit{Dendrobium} Madame Thong-In, respectively in \textit{A. tumefaciens} culture for 30 min. Tobacco nurse cells were added to acetosyringone containing medium during co-cultivation of \textit{Oncidium} explants (Liau \textit{et al.}, 2003). Ten hour co-cultivation of suspension cells of phalaenopsis was carried out with acetosyringone-activated \textit{A. tumefaciens} (Belarmino and Mii, 2000). During the current investigations, \textit{A. rhizogenes} culture was not pre-treated with acetosyringone. However, Liau \textit{et al.} (2003) pre-activated \textit{A. tumefaciens} culture with acetosyringone-activated \textit{A. tumefaciens} culture before infecting \textit{Oncidium} explants.

5.2.2.5 Co-cultivation time

After bacterial infection for 15 min, the cultures were transferred to basal MS medium with or without acetosyringone for co-cultivation for two days. In a similar way, explants of \textit{Dendrobium} Sonia were co-cultivated with \textit{A. tumefaciens} for two days (Pirla \textit{et al.}, 2005). Men \textit{et al.} (2003) suggested that higher co-cultivation time enhanced the transformation efficiency; the explants of \textit{D. nobile} were co-cultivated for two days by them. However, in \textit{Phalaenopsis} orchid transformation, extending the co-cultivation
period up to 5-7 days did not increase GUS transient expression activity and caused necrosis and death of cells (Belarmino and Mii, 2000). Necrosis and death of the infected PLBs was observed even in the two days co-cultivation time in the present species, indicating that the species was very fragile and needed to be handled with utmost care. Men et al. (2003) stated that exposing the infected PLBs immediately to the selection pressures could prevent the regeneration of chimeric or plants escaped from selection pressure; hence they only co-cultured for 2-3 days, before the PLBs were transferred to selective medium.

The co-cultivation time has been varied by earlier workers in order to enhance the transformation efficiency. Dendrobium Madame Thong-In explants were co-cultivated for 3 days with A. tumefaciens on antibiotic-free medium, which was followed by the second stage of co-cultivation of explants with slowly growing A. tumefaciens for a period of 3-4 weeks on medium containing 50 mg l⁻¹ carbenicillin. As one of the most important factors contributing to the transformation efficiency, the co-cultivation period was balanced between a maximal gene transfer and a minimal explant necrosis by manipulating the Agrobacterium concentration in the co-cultivation medium. However, a severe necrosis of explants caused by A. tumefaciens contamination was observed when the second stage of co-cultivation exceeded 5 weeks (Yu et al., 2001). Belarmino and Mii (2000) carried out a two-step co-cultivation of phalaenopsis suspension cells, i.e.; a 10 h co-cultivation of suspension cells in a suspension of A. tumefaciens, immediately followed by 3 days co-cultivation in 0.8% agar-solidified co-cultivation medium containing acetosyringone (500 µM). Two stages of co-cultivation method was also followed by Liau et al. (2003) for Oncidium transformation, where the first stage occurring on antibiotic-free medium for 3 days, and the subsequent stage on medium containing timentin (100 mg l⁻¹) for one month. After 10 h incubation in bacterial culture, Oncidium explants were co-cultivated for 3 days in dark in medium containing acetosyringone (200 µM) and a nurse culture of tobacco suspension cells (Liau et al., 2003).

5.2.2.6 Photoperiod

The infected explants of Malaxis acuminata were subjected to a photoperiod of 12 h under cool fluorescent light. Yu et al. (2001) conducted the co-cultivation of Dendrobium
Madame Thong-In explants under a 16 h photoperiod. Liau et al. (2003), however, carried out co-cultivation of Oncidium explants in dark

5.2.2.7 Acetosyringone

Activation of the vir genes of Agrobacterium rhizogenes by acetosyringone, a phenolic compound, is known to be essential for Agrobacterium-mediated transformation of plants. It was even supplied to the explants of the dicotyledonous species and some monocotyledonous species, where it was not essential for Agrobacterium based transformation, leading to a dramatic enhancement of the transformation efficiency (cf. Men et al., 2003). Men et al. (2003) demonstrated that acetosyringone increased the transient expression efficiency of the gus gene in Dendrobium nobile. Acetosyringone was supplied to the explants in MS medium, during their inoculation with the bacterial suspension as well as two days co-cultivation period, at a concentration of 100 μM.

In agreement with the present study, Men et al. (2003) and Pirla et al. (2005) added acetosyringone (100 μM) to the infected explants of D. nobile and Dendrobium Sonia for transformation with A. tumefaciens. However, higher concentration of acetosyringone has been employed to enhance the transformation efficiency in Oncidium (200 μM) (Liau et al., 2003) and Phalaenopsis (500 μM) (Belarmino and Mii, 2000). Another technique has been employed for the enhancement of transformation efficacy with A. tumefaciens in orchids. Acetosyringone-activated A. tumefaciens has been utilized for infection in Oncidium (Liau et al., 2003) and Phalaenopsis (Belarmino and Mii, 2000). Liau et al. (2003) obtained a few transgenic plants after A. tumefaciens transformation without acetosyringone treatment in the co-cultivation medium, however, the addition of acetosyringone increased the reproducibility of their protocol; implying that signal molecules produced by Oncidium orchid tissue might not be sufficient for the activation of A. tumefaciens vir genes. In the present study, the explants were not given any prior treatment with acetosyringone. However, the protocorms of Phalaenopsis orchid were cultured on acetosyringone containing medium 2 days before infection with A. tumefaciens (Mishiba et al., 2005).
Discussion

5.2.2.8 Medium for experiment and antibiotic treatment

After co-cultivation of two days, the explants were washed in sterile distilled water, blot-dried and placed on fresh MS medium containing augmentin (300 mg l\(^{-1}\)) for fifteen days. After 15 days, they were shifted to fresh medium containing the same concentration of augmentin. The augmentin treatment continued till three or four sub-cultures. The cultures were maintained for as long as possible, sometimes till four months. Men et al. (2003) used hygromycin and cefotaxime for selection of *D. nobile* transformed cultures. Carbenicillin (50 mg l\(^{-1}\)) was used in *Dendrobium* Madame Thong-In (Yu et al., 2001). Liau et al. (2003) used timentin and cefotaxime (at 50 and 100 mg l\(^{-1}\) concentrations, respectively) for eradicating the bacterial cells and selection of transformed *Oncidium*.

5.2.2.9 Response of explants

No root formation occurred till three or four months of culture of *Malaxis acuminata* explants, till the time the explants survived. Most of them underwent necrosis after a few sub-cultures. The infected explants were maintained for as long as possible. A few of them showed PLB multiplication in the medium. As per our knowledge, no report has been encountered of an orchid transformation with *A. rhizogenes*. However, positive results of transformation of orchids using *A. tumefaciens* have been reported; *Dendrobium* Sonia (Pirla et al., 2005); *D. nobile* (Men et al., 2003); *Dendrobium* Madame Thong-In (Yu et al., 2001); *Oncidium* (Liau et al., 2003); and *Phalaenopsis* orchid (Belarmino and Mii, 2000; Mishiba et al., 2005). A maximum transformation efficiency of 18% was obtained in *D. nobile* by Men et al. (2003), which was much higher than that obtained by other workers. The highest transformation efficiency (1.3-1.9%) was obtained in *Phalaenopsis* orchid. This low percentage of transformation was obtained despite pre-treatment of the protocorm explants in acetosyringone containing medium 2 days before bacterial infection (Mishiba et al., 2005).

All the above mentioned examples of successful transformation were carried out using *A. tumefaciens*. It is also noteworthy that these workers made use of only epiphytic orchid orchids whereas in the present set of investigations, the species utilized for transformation experiments was a terrestrial one. It could be concluded that the terrestrial
species', in the present case *Malaxis acuminata*, are more recalcitrant to transformation using *Agrobacterium*, in general, and *A. rhizogenes*, in particular.

Future experiments with *M. acuminata* should be carried out with an increased infection period of up to 30 min; prior co-culture of the explants with medium containing acetosyringone; increasing the concentration of acetosyringone; increased co-cultivation time, introducing a two-stage co-cultivation protocol; and other strains of *A. rhizogenes* might be tried; in addition to activating the bacterial cells with an appropriate concentration of acetosyringone. Stage and age of the explant at which the infection of the bacteria was carried out might also influence the efficiency, as was the case in the study by Mishiba *et al.* (2005) where immature protocorms of *Phalaenopsis* were targeted for transformation using *A. tumefaciens*.

5.3 SOLASODINE CONTENT IN SOLANUM XANTHOCARPUM

Though there have been reports of studies on the alkaloid production in wild plants and *in vitro* raised tissues of *Solanum xanthocarpum*, these have been meagre. The present sets of studies were designed to assess the variations in the formation of solasodine in the different parts of the wild plants *i.e.* stems, leaves, roots and also at the different stages of fruit development. The studies also aimed to estimate the effects of different types of *in vitro* raised tissues on the alkaloid production, in addition to assessing the effect of various plant growth regulators and hairy roots.

5.3.1 Variation in the solasodine content in different parts of the field grown plants

Various authors have studied the variation in the production of alkaloids in the different parts and physiological ages of plants of Solanaceae members and the factors affecting their formation.

5.3.1.1 Effect of stages of berry development on alkaloid production

The fruits were tested for solasodine content at three different stages of growth (very young green, 0.2-0.5 cm wide called 'a'; slightly mature green fruits, 0.6-1.0 cm wide called 'b'; and mature green fruits, 1.0-1.5 cm wide called 'c'). It was found that
the green fruits of size 'b' contained the highest solasodine concentration (0.991/1.0%) followed by 'c' (0.868%) and 'a' fruits (0.696%). The results indicated that out of the different ages of fruits, green fruits had the highest solasodine content followed by mature green and very young green fruits.

The present studies were designed to assess the effect of maturity and age of berries on the solasodine content and showed that solasodine content increased from very young berries till the stage of maturity; once the maturity was attained the solasodine content receded. Similar results were obtained in *Solanum khasianum* (Chaudhari and Hazarika, 1996; Saini, 1966) and *S. xanthocarpum* (Varghese and Sharma, 1983). Earlier, Willhun (1967) reported a decrease in the steroidal alkaloid content in the fruits of *S. dulcamara* with maturity. Out of the extracts of ripe, green-pale or green-yellow and yellow or red fruits, solasodine was found to be highest in ripe green-pale fruits in *S. americanum* and white yellow or red mature fruits had maximum soalsodine in *S. atropurpureum* (Saraiva de Siqueira *et al.*, 1988). Solasodine increased from 0.655% in *S. ciliatum* dry fruits to 2.90% in green-pale or green-yellow fruits and reduced to 2.09% on further maturity in yellow or red fruits (Saraiva de Siqueira *et al.*, 1988). These results were analogous to those of the present study in the case of *S. americanum* and *S. ciliatum* while opposite trend was observed in *S. atropurpureum*. However, the results of Siddiqui *et al.* (1983) were a complete contrast, with the unripe berries of *S. xanthocarpum* containing a higher concentration of solasodine (0.75 and 1.7% in ripe and unripe berries, respectively). Chand *et al.* (1995) showed that very young berries and very small leaves had higher contents of solasodine in *S. sisymbrifolium*.

One per cent (0.991%) solasodine was obtained from the mature green berries of *S. xanthocarpum* in the present study. Goswami *et al.* (1986) reported 0.996% and 0.84% solasodine from *S. xanthocarpum* green and ripe berries, respectively. Solasodine (987 mg 100 g⁻¹ DW) was found in the berries of *S. surattense* (Mazumdar, 1984). 1.7% solasodine was reported from the berries of *S. adoense* (Ntahomvukiye *et al.*, 1983); whereas 0.67% solasodine was isolated from the fruits of *S. paludosum* (Bhattacharyya, 1984). Fruits of *S. asperum* yielded only 0.24% solasodine (Bhattacharyya, 1984).
5.3.1.2 Variation in alkaloid production in different plant parts

During the course of the present study, stems, leaves, roots and fruits had solasodine in an ascending order (0.083, 0.192, 0.205, and 0.991 per cent solasodine in stems, leaves, roots and fruits of field plants of Solanum xanthocarpum) showing that the fruits contained a remarkably higher percentage of the alkaloid than other plant parts (Fig. 394). Leaves, stems and roots were harvested at the reproductive stage and leaves were harvested irrespective of their size. Similarly, Goswami et al. (1986) showed that the fruits of S. spirale contained almost twice the amount of solasodine than that present in the leaves (0.06 and 0.12%, respectively). Cham and Wilson (1987) analysed the solasodine contents of S. sodomaeum and found that ripe fruit had the highest contents, followed by unripe fruit, leaves, and stems. In consonance with the current study, Subramani et al. (1989) found that in S. sisymbrifolium, fruits and leaves followed a descending pattern (0.93, 0.73, and 0.23%, respectively) of solasodine production. However, leaves and berries of S. trilobatum contained the highest and the lowest amount of solasodine, respectively (Balakrishna et al., 1992). Maximum concentration of steroids was observed in leaves during flowering after which it decreased (Muravéva et al., 1969). Diosgenin was reported to be highest in ripe berries followed by green berries and stems in S. incanum (Ntahomvukiye et al., 1981). Similar results have been reported in different Solanum species (cf. Jaggi and Kapoor (1997) where fruits have been reported to be the richest source of solasodine. Roots contained high amounts of solasodine in the present study indicating its synthesis de novo in the roots and its transport to leaves for storage. Similarly, Sangwan et al. (2008) stated that root-contained withanolide A is de novo synthesized within roots from precursors in Withania somnifera; and that withanolides were synthesized in roots and transported to leaves for storage. The results of Iranbakhsh et al. (2006) showed that alkaloid production (atropine and scopolamine) in Datura stramonium started from the end of the second week after seed germination, increased in different organs up to the tenth week of growth, and then decreased. Leaves and capsules showed the highest alkaloid content in the vegetative and generative stages, respectively. In leaves, the alkaloids decreased rapidly in the generative stage. The highest alkaloid content was recorded in vegetative leaves, followed in descending order by vegetative petioles,
Discussion

generative and vegetative stems, generative petioles, generative roots, generative leaves, vegetative roots and mature seeds.

The solasodine content in the different plant parts of Solanum xanthocarpum were tested colorimetrically to determine the variation in the content in the different parts. The results indicated that out of the plant parts green fruits had the highest solasodine content followed by mature green and very young fruits. Roots, leaves and stems followed in descending order, thereby indicating that the fruits were the richest source of solasodine.

In our study, it has been clearly indicated that the content was high in green fruits while it decreased on further maturation of the fruit (0.991 and 0.868% solasodine in ‘b’ green fruits and ‘c’ mature green fruits, respectively). While at the same time, leaves and roots contained 0.192% and 0.205% solasodine indicating a transport of the alkaloid from leaves and roots to the fruit pulp. The subsequent decline in the solasodine yield after fully developed stage of berries pointed either towards migration of solasodine glycosides to other plant organs or their degradation/conversion to other compounds. In a similar study, Varghese and Sharma (1983) pointed out that the content was high in the early stages of berry development which declined on maturation in S. xanthocarpum. The yellow fruit contained the maximum solasodine in its fruit pulp at the age of 80-90 days after fertilization on per berry basis. The results suggested the transport of solasodine from leaves and berry wall to the fruit pulp. The mucilaginous part constituted the bulk of solasodine present in the berries at all the stages of development suggesting its accumulation in the mucilage that surrounds the seeds. A similar finding was earlier reported by Saini (1966) in S. khasianum. Chaudhari and Hazarika (1996) showed that the maximum extractable solasodine contents occurred in the green to yellow mature berries of S. khasianum and on further maturation (deep yellow berries), the contents reduced. However, it has also been reported that S. xanthocarpum contained maximum solasodine in the berry wall (cf. Jaggi and Kapoor, 1997). A similar study was conducted on S. sisymbriifolium and S. xanthocarpum by Chand et al. (1995). While in the former species maximum solasodine contents were shown by berries in their very young stage (0.26%) and by leaves in their very small stage (0.16%); the latter taxon showed maximum solasodine contents in mature coloured stage of berries (1.74%) and very small
stage of leaves (1.04%). Decreasing trend in solasodine content with increase in solasodine yield between very young and fully developed stages of berries showed that the rate of solasodine biosynthesis lags behind the rate of dry weight accumulation but afterwards same moves ahead to the rate of dry weight production till mature coloured stage. A similar study shows the translocation of cholesterol from the leaves to ripening fruits of *S. khasianum* (David Nes et al., 1982).

The solasodine content in *S. sisymbriifolium* increased with the percentage of the berries reaching its maximum value (7.2% of dry weight) 45 days after anthesis when the colour was scarlet. Thereafter the content decreased upon wrinkling of the berries (4.2%). The size of the berries and moisture content showed a direct correlation with solasodine. In leaves, the solasodine content varied from 6.4-7.2%. Total nitrogen content of berries decreased with age and the total sugars gradually increased parallel with the solasodine content. The observation supports the fact that nitrogen fertilizers decrease the level of glycoalkaloids in *Solanum*. This may be due to the fact that high nitrogen dressings cause the plant to be depleted of carbohydrates which are the source of carbon, both for the aglycone and the glucoside part of the glycoalkaloid (Pandeya et al., 1980).

All tested organs of *S. nigrum* and *S. incanum* elaborated solasodine, but the levels varied widely. In both species, the smallest leaves showed the highest alkaloid concentration. Maximum levels in *S. incanum* leaves were greater than twice those in *S. nigrum*. The absolute amount of alkaloid per leaf increased during leaf development whereas the concentration declined. The pattern of change in leaf alkaloid concentration with increasing age of the plant was different in the two species. The concentration of alkaloid in roots was higher than in the stem in *S. nigrum*, but initially similar to stem levels in *S. incanum*. Both organs showed alkaloid accumulation with time, although root levels inexplicably fell in *S. incanum* after 20 weeks. Small *S. nigrum* unripe fruits had a high concentration of solasodine, but both the concentration and the absolute amount per fruit decreased with fruit maturation. The concentration and absolute amount of alkaloid also diminished in fruits of all developmental stages with increasing age of the plant (Eltayeb et al., 1997).
Discussion

What is significant for the biosynthesis of solasodine is that the fruits, which are the richest source of the alkaloid, import cholesterol from the leaves (David Nes et al., 1982). Increasing water stress induced an increase in the amount of solasodine in the mature berries of *S. khasianum*. Since cholesterol is the precursor of steroidal alkaloids, its biosynthesis, translocation, and metabolism are involved in this effect; water stress could stimulate cholesterol production, its translocation to the sites of alkaloid synthesis, and its transformation into the alkaloids. Cholesterol originally present in the leaves was translocated to fruits; if solasodine synthesis were restricted to the fruits, the imported cholesterol would contribute to solasodine production. The absence of labeled cholesteryl glycosides indicate that glycosidation of cholesterol does not precede side-chain hydroxylation in the formation of glycoalkaloids (cf. David Nes et al., 1982).

**Fig. 394 Variations in the percentage of solasodine in different plant parts of *in vivo* Solanum xanthocarpum**

All the plant parts were tested for solasodine levels, as it is not possible to estimate a plant’s biosynthetic capacity without analyzing the whole plant. Solasodine in the fruits of different ages showed a marked difference in magnitude of accumulation. Therefore, in order to know the percentage of the alkaloid present in a species, it is important to test the various plant parts and at different stages of growth and development to get a wholesome view and data about the per cent alkaloid present in a particular species. Even in a single species, the different seasons and stages of
Development play an important role in the percentage of the alkaloid that would be available at that particular time period.

5.3.2 Variation in the solasodine content in *in vitro* raised organs

The concept of morphogenetic and biosynthetic totipotency has been demonstrated in several plant tissue culture systems and it is generally accepted that plant cells in culture possess all the genetic information of the parent plant. However, during the initiation of tissue cultures from an organized explant; several biochemical, physiological and cytological changes take place. On subculture, these changes may persist or lead to further changes and as a result, the behaviour of tissue culture systems is often unpredictable. These events may have strong influence on the biosynthetic potential of the tissues and thus may result in change in biosynthetic products both qualitatively and quantitatively (Kumar, 1992).

Solasodine was obtained from the multiple shoots, calli and roots growing in different medium combinations in the present study. Solasodine has been earlier reported in tissue cultures in *Solanum lacinatum* (Chandler and Dodds, 1983), *S. sarrachoides* (Banerjee *et al.*, 1993), *S. trilobatum* (Krishnamurthy and Prabia, 1995), *S. xanthocarpum* (Heble *et al.*, 1971), *S. verbascifolium* (Jain and Sahoo, 1981). Sapogenin and solasodine was isolated from *in vitro* cultures (undifferentiated callus tissue) of some solanaceous plants (*S. aviculare*, *S. elaeagnifolium*, *S. khasianum*, *S. nigrum*, and *S. xanthocarpum*) (Khanna *et al.*, 1976). Soladulcidine, tigogenin and diosgenin were isolated from the callus cultures of *S. dulcamara* in addition to solasodine (Emke and Eilert, 1986).

The tissue cultured seedlings grown in basal MS medium, callus growing in MS+IBA<sub>4.4</sub> and multiple shoots growing in MS+BAP<sub>4.8</sub> had lower solasodine than ‘b’ fruits, respectively. None of the tissue culture and plant growth regulator treatments were able to match the solasodine contents found in the fruits. However, the callus growing in the MS medium containing IBA at 4.4 μM concentration were able to match up to the solasodine content found in the field grown plant’s roots and the roots procured from medium enriched with NAA at 4.4 μM produced more solasodine than that found in the field grown leaves, stems and roots.
5.3.2.1 Effect of plant growth regulators in the nutrient medium

In the present study, BAP at 8.8 μM concentration in full strength MS medium containing 3% sucrose showed solasodine content which was comparable to that present in the field grown plant’s leaves and higher than that of the field stems. In a similar study, Bhatt et al. (1983) reported a significant increase in solasodine production in S. nigrum when sucrose concentration was increased to 4, 6, and 10% level in the medium containing 10.0 μM BAP as the sole growth regulator.

During the current taxon, the results showed that NAA led to a decline in solasodine accumulation in the callus cultures. Also an increase in the concentration from 2.2 to 4.4 and 8.8 μM caused a further reduction in the alkaloid levels. In consonance with our results, it was shown that NAA repressed anthocyanin production in callus cultures of Oxalis linearis with an increase in NAA from 8.0-32.0 μM (Meyer and van Staden, 1995). However, Khanna et al. (1976) concluded that NAA treated callus of Datura metel had the maximum alkaloid in comparison with that of grown in IAA and 2,4-D containing media. Zheng and Zheng (1976) illustrated the stimulating effect of NAA on the production of scopolamine in the callus cultures of Scopolia acutangula. The roots obtained from medium enriched with NAA (4.4 μM) led to a noteworthy increase in solasodine percentage in the present study and the percentage was higher than that in the leaves, stems and roots of the field grown plants. Similarly, Niño et al. (2003) reported the enhancement of scopolamine formation in root cultures of Brugmansia candida on the addition of 3.0 mg l\(^{-1}\) NAA to the medium. Piñol et al. (1984) demonstrated that in the presence of a constant amount of kinetin, it is the auxin concentration in the culture medium that controls the rate of nicotine synthesis in the callus cultures of Nicotiana tabacum. Shimomura et al. (1987) reported the presence of atropine and scopolamine in the adventitious root cultures of Duboisia hybrid on MS medium supplemented with IAA (1.0 mg l\(^{-1}\)) and NAA (3.0 mg l\(^{-1}\)). Singh (1995) reported an increase in solasodine synthesis in the leaf cultures of S. platanifolium when grown in MS medium supplemented with BAP (2.0 ppm) and NAA (1.0 ppm). Khanam et al. (2000 b) assessed the contents of hyoscyamine and scopolamine in calli and non-rooted shoots obtained from calli of Duboisia myoporoides and found that BAP (10.0 μM) + NAA (1.0 μM) in the regeneration medium helped form the above alkaloids.
**Discussion**

The calli growing in MS+2,4-D at 4.4 and 8.8 µM concentration produced about one-third and half when compared to solasodine produced by the tissue cultured seedlings in basal MS medium (0.078, 0.057, 0.116% in calli growing in MS+2,4-D at 4.4 and 8.8 µM concentration and tissue cultured seedlings in basal MS medium, respectively). Similar studies were also carried out on the unorganized suspension cultures of *Costus speciosus*, *Dioscorea floribunda*, *Solanum aviculare* and *S. xanthocarpum* and a significant increase in diosgenin in all the species was recorded whereas a many fold solasodine content was observed in suspension culture of *S. aviculare*. However, 2,4-D was more effective than IAA, NAA, KN or BAP in stimulating solasodine production in cultures of *S. indicum*, *S. khasianum* and *S. surattense* (Barnabas et al., 1989). Nigra et al. (1987, 1989) advocated the use of 2,4-D for higher solasodine yield in tissue cultures and calli of *S. eleagnifolium*.

Heble et al. (1971) stated that the actively growing tissues in culture were influenced by auxins, kinetin, and gibberellic acid in their biosynthetic potential. It is possible that certain functions are reduced or simplified under cultural conditions while others are enhanced, as shown by the absence of solasodine and the augmentation of diosgenin with the exogenous supply of IAA or IBA in the medium. However, changes in the tissue morphology did not show any concomitant changes in the production of other secondary metabolites. *S. laciniatum* tissue cultures produced diosgenin (quantity being identical with that of the field plants) but the main steroid, solasodine was not observed (Vágújfalvi et al., 1971).

Presently, when callus growing in medium fortified with IBA was tested for its solasodine content, 4.4 and 8.8 µM concentrations produced 0.324% and 0.286% solasodine, respectively both of which were higher than that found in the leaves, stems and roots of the field grown plants of *Solanum xanthocarpum*. However, no solasodine was present in *S. xanthocarpum* tissue cultures grown on medium containing IAA or IBA (Heble et al., 1971). Hashimoto et al. (1987) also reported reduced alkaloid biosynthesis in *Hyoscyamus albus* and *H. niger* root cultures in the presence of IBA.

In the in vitro organs, roots produced the maximum percentage of solasodine followed by callus and multiple shoots in descending order. The highest solasodine was observed in the roots growing in medium containing NAA (4.4 µM, 0.579%) followed by
calli in IBA enriched medium (4.4 and 8.8 μM; 0.324% and 0.286%, respectively) and multiple shoots procured from medium supplemented with BAP (8.8 μM, 0.199%).

This showed that the plant growth regulators work singly or in synergism and indicated chemical regulation by auxins. In the present study, it was indicated that the content of solasodine changed on addition of various growth regulators, in addition to the variation observed in the various tissues. Similarly, Heble et al. (1971) analyzed the tissue cultures of *S. xanthocarpum* subjected to the influence of different hormones such as 2, 4-D, IAA, IBA, KN, and GA singly and in synergistic combinations and showed changes in the steroidal content indicating regulation by auxins. Similar sentiments were also expressed by Jaggi et al. (1990). However, in a study by Jaggi et al. (1987), it was shown that different growth regulators used in various concentrations could not change the pattern of secondary metabolites produced in *S. xanthocarpum*. In contrast, our results indicated IBA to be the most beneficial in promoting solasodine production in callus cultures closely followed by KN and BAP; BAP was the most useful in content enhancement in the case of multiple shoot cultures; while NAA was optimal for enhancing the alkaloid content in in vitro roots. Khanam et al. (2000 a) stated that the tropane alkaloid biosynthetic ability of the non-rooted shoots of *Datura myoporoides* depended on the cytokinin/auxin combinations used at the callus induction stage. Jayraaj and Saroja (2006) studied the influence of auxins and cytokinins on growth and alkaloid production in *Adathoda* species *in vitro*. Among the auxins tested (IAA, NAA, IBA, 2, 4-D), IAA was able to increase alkaloid production in callus cultures. Of the two cytokinins used, KN enhanced the production of alkaloid. Both the set of results were, however, contradictory to the present results, where NAA as well as KN gave poorer alkaloid production than IBA enriched medium.

### 5.3.2.7 Effect of increasing concentrations of growth regulators

During the course of the present investigations, it was observed that an increase in the concentration of the growth regulator IBA led to a diminution in solasodine percentage in the calli. In the present study, the results showed that NAA led to a decline in solasodine accumulation in the callus cultures; also an increase in the concentration from 2.2 to 4.4 and 8.8 μM caused a further reduction in the alkaloid levels. Though 2, 4-
D caused better solasodine content than NAA in the calli, an increase in its concentration from 4.4 to 8.8 µM also led to a decrease in solasodine concentration. Similarly, an increase in BAP concentration from 4.4 to 8.8 µM led to a reduction in solasodine concentration extracted from the calli. However, the increased KN concentration of 8.8 µM promoted a slight increase in the alkaloid concentration in the calli. In consonance with our results, it was shown that medium combination containing BAP and IAA both at a concentration of 2 ppm each decreased the content of solasodine in tissue cultures of *S. platanifolium* compared to that found in medium supplemented with BAP (2.0 ppm) and NAA (1.0 ppm). However, a reverse trend was observed in the case of multiple shoots where higher concentration (8.8 µM) of both BAP and KN (alone) was more beneficial in enhancing the solasodine content. In the case of in vitro roots, lower concentrations (4.4 µM) of NAA and BAP promoted more solasodine production than their higher (8.8 µM) concentrations; whereas higher concentration of KN (8.8 µM) produced more solasodine. Roots growing in NAA also produced lower amounts of solasodine when its concentration was increased from 4.4 to 8.8 µM causing a two fold reduction. This shows that auxin excess could lead to a reduction in the alkaloid formation. Hashimoto *et al.* (1986) reported a similar decrease in alkaloid biosynthesis in *Hyoscyamus albus* and *H. niger* root cultures with an increase in the concentration of auxins. Earlier workers have also noted that an excess of auxin has led to dedifferentiation of the hairy roots or to callus formation (Palazon *et al.*, 1995; Paniego and Giulietti, 1996). Jayraaj and Saroja (2006) found that higher concentration of auxin (2.0 mg l⁻¹) was the best for maximum alkaloid production in *Adathoda* species (Acanthaceae).

Pandhair *et al.* (2006) concluded that the varietal variation, explant sources and composition of the culture medium determined the capacity of *Capsicum annuum* callus cultures to synthesise capsaicin. It was found that nutrient limitation enhanced the capsaicin accumulation in callus cultures. Nutrient limitation reduced the growth by decreasing the primary metabolism thereby, increasing the synthesis of secondary metabolites. Similarly, in general, lower concentrations of the growth regulators were beneficial in enhancing the solasodine content in the present taxon.
5.3.2.3 Effect of type of culture and organogenesis

In the present study, maximum solasodine content was observed in the in vitro roots (0.579%, NAA₄,₄) followed by calli (0.324%, IBA₄,₄) and multiple shoots (0.199%, BAP₈,₈). This showed that in vitro roots retained their biosynthesis capacity that was present in the in vivo roots. However, multiple shoot formation led to a decline in the alkaloid content.

Khanam et al. (2000 b) indicated that although root was the main site of alkaloid biosynthesis in Duboisia myoporoides, with suitable cell differentiation, alkaloid biosynthesis may take place in cultured shoots without root initiation. This observation was similar to that of the present study where multiple shoots produced considerable amounts of solasodine even without root formation.

Presently, it was observed that in vitro seedlings produced a very minute quantity of solasodine. Similar findings were reported in the in vitro seedlings of Solanum platanifolium (Singh, 1994).

The multiple shoots in the present taxon have shown solasodine content similar to that of the field grown leaves only in the case of BAP at 8.8 μM. Multiple shoots are photo-autotrophic tissues and were incubated under light conditions. The study by Conner (1987) showed that Solanum lacinatum shoots regenerated from callus and cultured under heterotrophic conditions showed lower levels, while shoots cultured photoheterotrophically or photoautotrophically yielded solasodine concentration approaching those of the field grown plants. Solasodine biosynthesis is promoted by actively photosynthesizing chloroplasts, and cell cultures yield only low solasodine levels as a consequence of their heterotrophic mode of nutrition (Conner, 1987). However, dark grown cultures of S. lacinatum contained significantly more solasodine than those incubated in light conditions (Chandler and Dodds, 1983).

In the present study, callus formation led to higher solasodine content than the multiple shoots. This was, nevertheless, in contrast to earlier reports where organogenesis led to higher alkaloid production in in vitro cultures. Solasodine concentration was enhanced by the induction of organogenesis of both primary leaf explants and callus in S. lacinatum cultures (Chandler and Dodds, 1983). In most Solanum cell cultures studied so far alkaloid accumulation was enhanced by organogenesis (Roddick and Butcher, 1972;
Bhatt et al., 1983; Chandler and Dodds, 1983). A cytological examination of the callus tissue of *Nicotiana tabacum* showed that the greater degree of cellular differentiation which causes the loss of meristematic areas, inhibited alkaloid synthesis (Piñol et al., 1984). Emke and Eilert (1986) found that greening and organogenesis promoted alkaloid accumulation. This resembles the situation reported in potato tubers where greening and sprouting induced alkaloid formation (Sinden et al., 1984). It was reported by Bhatt et al. (1983) that the tissues grown in darkness the differentiated tissues produced significantly more (1.5-1.0 times) solasodine than the callus. Singh (1994) studied the effect of organogenesis on solasodine content in *S. platanifolium* and concluded that organogenesis helped enhance the content, with *in vitro* seedlings and undifferentiated calli failing to produce any alkaloid; establishing a direct correlation between solasodine content and differentiation. All callus cultures procured from different parts of *in vitro* seedlings except undifferentiated leaf callus, produced solasodine but these seedlings as such failed to produce any solasodine. Differentiated callus culture of *S. platanifolium* leaf showed the highest solasodine concentration compared to that from stems, hypocotyls and cotyledonary leaves (Singh, 1995). Molecular differentiation, cellular differentiation and organ differentiation influence product biosynthesis (Heble, 1996). Regenerated shoots of *S. laciniatum* yielded higher solasodine content than undifferentiated as well organogenic callus; being ten times higher than the callus culture and approaching the amount observed in the field grown plants (Bhatnagar et al., 2004). The alkaloid profile in cultures of *Nothapodytes foetida*, an anti-cancer drug yielding camptotheca alkaloids, indicated a relationship between organogenesis and production of camptotheca alkaloids (Raul and Heble, 2003). Results suggested that production of withanolides was closely associated with morphological differentiation in *Withania somnifera* cultures (Sharada et al., 2007).

Presently, *in vitro* roots produced much higher solasodine than that produced by the calli. In consonance with these results, Hashimoto et al. (1986) reported that higher hyoscyamine and scopolamine content in cultured roots than the amount produced in the callus of *Hyoscyamus* species.

Unorganised tissue cultures often lose the ability to produce major secondary metabolites characteristic of the parent plant. This loss of ability, however, can be
Discussion

restored if the callus is differentiated into shoots or roots or regenerated into a plantlet. Induction of organogenesis in callus or cell cultures has been able to increase the yield of active constituents (Singh, 1994).

Petri and Spassova (1989) showed an increase in the atropine and scopolamine contents in the rooted regenerants of *Datura innoxia*, thereby showing that the callus cultures retained their genetic information for the synthesis of alkaloids but their juvenile stages were not conducive to alkaloid production; there was somaclonal variation of secondary metabolite production; and root organization played a role in the synthesis of tropane alkaloids. Hiraoka and Tabata (1974) also indicated the association of root formation in scopolamine production in *Datura innoxia* cultures. They demonstrated that synthesis of tropane alkaloids was promoted significantly in the roots differentiated from callus tissues, showing a tendency towards recovery of normal chemical pattern with differentiation. Tissue culture technique has a limitation that generally many cultures do not produce significant amounts of the compounds characteristic of the plant from which they are derived. Probably in many cases this failure may be due to the reason that some secondary products are produced as a consequence of the special physiological and morphological properties of cultured tissues and cell differentiation helps in producing equal or sometimes increased amounts of secondary metabolites (Singh, 1994).

The alkaloid content varies with the type of tissue being used as the explant, the different types of organ cultures also lead to varying amounts of the alkaloid.

5.3.2.4 Effect of light on solasodine content

During the current investigation, the cultures were invariably grown under light conditions (16/8 h L/D). Reports on the influence of light on steroidal alkaloid concentration are contradictory. Chandler and Dodds (1983) observed an inhibitory effect on solasodine accumulation in *S. laciniatum*, whereas in *S. nigrum* callus solasodine concentration increased under a 16 h photoperiod (Bhatt *et al.*, 1983).
5.3.2.5 Comparison between the solasodine content in field plants and tissue cultured plants

In the present study, multiple shoots growing in BAP (8.8 μM) enriched medium produced higher solasodine content (0.199%) than that found in the leaves and stems of field plants (0.192 and 0.082%, respectively). The calli formed in IBA supplemented medium (4.4 μM) promoted the synthesis of solasodine (0.324%) which was higher than that found in the leaves, stems as well as roots (0.205%) of the in vivo plants. When the percentage of solasodine produced by in vitro roots was assessed, NAA (4.4 μM) containing medium was favorable for producing more than double amounts of the alkaloid than those produced by in vivo roots. The tissue cultured seedlings were, nonetheless, not able to match up to the amount of alkaloid found in the in vivo plant parts. However, none of the tissue cultured organs were able to reach the percentage of solasodine produced by the in vivo fruits (Fig. 395). These results indicate that suitable growth regulator combination and stage of growth was essential in enhancing the alkaloid production in tissue culture. Zheng and Zheng (1976) reported Scopolia acutangula calli to produce hyoscyamine and scopolamine in lesser quantity than the stems of the intact plant which was disparate to the present study where calli produced solasodine in higher amounts than that of the in vivo stems as well as leaves.

In the present study, multiple shoots were able to form comparable amounts of solasodine to those found in the leaves and stems but lesser than those of in vivo roots; Kang et al. (2004) found in vitro raised plants of Scopolia parviflora to contain higher level of hyoscyamine and scopolamine than that in the field growing plants. The maximum accumulation of nicotine in Nicotiana tabacum callus was slightly higher than that in the intact plant when nicotine and KN+IAA were added to the medium (Piñol et al. 1984). Leaf derived callus cultures of Solanum laciniatum contained much less solasodine compared to the parent plant foliage (Conner, 1987). This was in contrast to the present study where leaf derived calli formed solasodine in elevated percentage than that in the leaves, stems and roots of in vivo plants. The results of Eapen et al. (1978) were also in contrast with the present study where the calli and young shoot buds of Atropa belladonna had very low alkaloid contents as compared to those present in the mature parent plant.
Kitamura et al. (1985) were unable to detect the main alkaloids in the leaves of regenerated plantlets of *Duboisia myoporoides* at the early stage of development, but eventually the alkaloid spectrum approached that of the mother plant at the later stage. The alkaloid pattern of *Atropa belladonna* plant and root cultures was investigated by Hartmann et al. (1986) and observed that the root cultures differed only quantitatively in the alkaloidal patterns with those of intact plants. In the current study, however, *in vitro* roots produced almost double the amount of solasodine than *in vivo* roots.

*Although the in vitro multiple shoots, calli, and roots of Solanum xanthocarpum were able to match up to the in vivo plant parts (leaves, stems, and roots) with respect to their solasodine contents, they were unable to produce the levels approaching those found in the in vivo fruits.* It was probably due to the fact that conditions in sterile culture were sub-optimal for alkaloid synthesis. Similar opinion was also voiced by Parr and Hamill (1987) in the case of some species of *Nicotiana*.

**Fig. 395 Variations in the percentage of solasodine in *in vivo* plant parts and *in vitro* organs of *Solanum xanthocarpum***

![Bar chart showing the percentage of solasodine in different plant parts](chart)

**5.3.3 Effect of *Agrobacterium rhizogenes* infection on the solasodine content in *Solanum xanthocarpum* hairy roots**

*It was observed in the present taxon that the control roots accumulated 0.008% solasodine which was lesser than that produced by the ‘b’ green fruits and that produced by the roots of the field plants. They contained lesser solasodine than that present in the*
Discussion

in vitro roots growing in MS medium containing NAA at 4.4 μM. The hairy roots contained even lower percentage of solasodine (0.00115%) which was lesser than the 'b' fruits, the control roots, the field roots and the roots grown in MS+NAA. The results indicated that the Agrobacterium treatment was unable to enhance the production of solasodine in the hairy roots of Solanum xanthocarpum.

The transformed root cultures were able to produce a little amount of solasodine (0.00115%) in the present study. Similarly, transformed root cultures of Artemisia annua established by infection with A. rhizogenes strain LBA 9402 showed a strong morphological instability and accumulated low levels of artemisin (0-0.01% dry weight) (Paniego and Giulietti, 1996). The lower levels of solasodine in the transformed roots could be due to the changes brought about in the levels of various hormones in the roots which determine or help in the biosynthesis of the alkaloids. It was observed in a study by Schmulling et al. (1993) that expression of a single rol gene of TL-DNA of strain A4 in transgenic Nicotiana tabacum and S. tuberosum plants altered the internal concentration of, and sensitivity to, several hormones. Levels of immunoreactive cytokinins, abscisic acid (ABA), gibberellins, and IAA were analysed in apical shoots, stems, leaves, roots, and undifferentiated callus tissue. The addition of dominant and morphogenetically active rolA, rolB or rolC genes resulted in alterations in the content of several hormones. rolC expression, in particular, led to an up to 4-fold increase in the content of isopentenyladenosine, dihydrozeatin riboside, and trans-zeatin riboside type cytokinins in potato plants.

The lower solasodine content in the hairy roots of the present taxon could be explained by the fact that hairy roots and normal roots grow in a different manner. Hairy roots are more highly branched and rapidly growing and thus the two systems cannot be equated. Some of the differences in alkaloid content of the two systems could also be attributed to the inherent differences in physiological age structure. We could also opine that the full biosynthetic capacity may not always be expressed in culture. In a similar tone, Parr et al. (1990) had pointed out that in the case of tropane alkaloid production by solanaceous species the conditions in culture may sometimes be unfavourable for the full expression of a line’s potential biosynthetic capacity.
Drewes and van Staden (1995) observed that solasodine accumulated in the hairy roots (126 μg g⁻¹ dry wt.) of *S. mauritianum* was lower than that recorded in the plant's green berries (184 μg g⁻¹ dry wt.), but was far higher than that recorded in the roots on an intact plant (30 μg g⁻¹ dry wt.). It was also higher than the level in callus or suspension cell cultures, making this method a favourable system for the potential *in vitro* synthesis of solasodine. In the present study, however, the hairy roots recorded an all time low than the tissue cultured as well as the various *in vivo* plant parts. The hairy root cultures were grown and maintained on MS medium supplemented with 3% sucrose. Drewes and van Staden (1995), however, added myo-inositol (0.1 g l⁻¹) in addition to 3% sucrose to MS medium. When the medium strength was reduced to half or 3% glucose was substituted by 3% sucrose, the levels of solasodine reduced and growth rate of roots reduced.

The lower solasodine production in the hairy roots as well as the untransformed roots *in vitro* in the present study could be due to the fact that the complete synthesis of many metabolites in plants requires the involvement of both the roots and the shoots. A precursor might be available in the roots and then translocated to the shoots/leaves for the conversion into another metabolite or, *vice versa*. If expression of the enzyme remains organ-specific in tissue culture, neither the transformed roots nor the normal roots would be able to synthesize the final compound/metabolite. Parr *et al.* (1990) similarly pointed out that in such cases transformed roots or shooty teratomas by themselves would not be capable of synthesizing the final product and quoted the effectiveness of interspecies and intergenus organ co-culture for the production of secondary metabolites. Co-cultures of *Atropa belladonna* transformed roots and *Duboisia* hybrid shooty teratomas produced significant levels of scopolamine, whereas no scopolamine could be detected in separate root and shoot cultures in a study by Mahagamasekera and Doran (1998). In a similar way Subroto *et al.* (1996) pointed out that co-culture mimics the whole plant by providing the opportunity for localized metabolite synthesis and translocation of compounds between organs for further bioconversion and proved to be an effective technique for improving tissue-specific secondary metabolite synthesis.

Some of the hairy roots showed a tendency of dedifferentiation in the present study. The hairy roots of *Artemisia annua* also exhibited a strong tendency toward dedifferentiation in liquid B₅ medium (Paniego and Giulietti, 1996). Their strategy to
reduce salt concentration in the medium was unsuccessful. The addition of anti-auxin (PCMP) caused redifferentiation, with an initial combination of differentiated and dedifferentiated tissues in the cultures. In the present study, two morphologies of hairy roots were observed; typical hairy roots and callus-like roots with slower growth. In a similar manner, Moyano et al. (1999) have reported two morphologies of hairy roots: typical hairy roots with high capacity to produce alkaloids and callus-like roots with faster growth capacity and lower alkaloid production which showed a clear relationship between root morphology and alkaloid production. However, in the present study, alkaloid extraction was not undertaken in the callus-like hairy roots. Earlier workers have noted that an excess of auxin led to dedifferentiation of the hairy roots or to callus formation (Palazón et al., 1995; Paniago and Giulietti, 1996).

In contrast to the present investigations, literature is replete with reports on higher alkaloid production in hairy root cultures than the control in vitro roots or in vivo plant parts [Atropa belladonna (Kamada et al., 1986), Datura candida (Christen et al., 1989), D. innoxia (Boitel-Conti et al., 1996, 2000), D. innoxia, Hyoscyamus albus, H. niger, and Scopolia tangerica (Shimomura et al., 1991 a), D. stramonium (Payne et al., 1987), D. stramonium and H. niger (Jaziri et al., 1988), H. albus (Sauerwein and Shimomura, 1991; Zehra et al., 1999), H. muticus (Oksman-Caldentey et al., 1994; Zehra et al., 1999), Nicotiana species (Parr and Hamill, 1987), Solanum aculeatissimum (Ikenaga et al., 1995), S. aviculare (Kittipongpatana et al., 1998; Subroto and Doran, 1994), S. eleagnifolium (Alvarez et al., 1994), S. khasianum (Jacob and Malpathak, 2005 a), and Withania somnifera (Kumar et al., 2005 a)].

Future experiments could be planned to enhance the alkaloid production in Solanum xanthocarpum hairy roots by using various strategies such as, altering the essential nutrients and their ratio like nitrogen and carbon; manipulating the nutrient medium; adding growth regulators and/or gibberellic acid to the medium; enhancing sucrose concentration; using another strain of Agrobacterium rhizogenes; co-culturing the transformed roots and shooty teratomas; incubating the hairy roots under light conditions; treatment with biotic and abiotic elicitors; feeding solasodine precursors; and subjecting the explants to prior conditioning.