Chapter 5

DISCUSSION
Maintenance of axenic conditions is an essential pre-requisitic aspect for a successful plant tissue culture experiment. Because the unsterilized media and explants support luxuriant growth of microorganisms (Bacteria and Fungi), which not only compete in nutrition with explants, but also kill them by their allelopathic chemicals released in medium.

It is an admitted fact that no experiment can get a headway unless at primary level one's hand first becomes set on overcoming the sterilization hurdles. Hit and trial with extra care can definitely help the researcher in achieving the desired goal. While persuing the literature, it was noticed that Tabachnick and Kester (1977) and Kester et al. (1986) in almond; Norton and Norton (1986) in Prunus cerasifera and Hammatt and Grant (1998) in Prunus serotina reported maximum sterilization of dormant buds and young shoots by using sodium hypochlorite (NaOCl) solution (0.5-10%) along with 0.1% Tween-20 for 10-20 min. In present studies after adopting similar procedure and instead using calcium hypochlorite Ca(OCl)₂ solution (7-10%) along with 0.05% Tween-20 for 20-25 min resulted in 10% sterilization of explant, which is not in consonance with the studies of Rugini and Verma (1982); Rugini and Monastra (1991): Anisley et al. (2000) in almond and Bouza (1997) in Prunus tenella, who reported maximum sterilization of shoot tips and nodal segments at 6-9% of Ca(OCl)₂ for 15-20 min.
In present studies 90.25% of explant (shoot tips) sterilization was achieved by using a solution containing HgCl₂ (0.2g) and NaOCl (1ml) per 100ml of double distilled water along with 0.05% Tween-20 for 25 min. These results contradict the earlier reports, where sterilization was achieved by using only one type of sterilent either NaOCl or Ca(OCl)₂ or HgCl₂ (0.1-0.2%) (Goudarzi et al., 1997 in Prunus avium; Bouza, 1997 in Prunus tenella; Hammatt and Grant, 1998 in Prunus serotina and Anisley et al. 2000 in almond).

The sterilization percentage achieved varied from treatment to treatment depending upon the plant material (dormant buds and young shoots), time of collection from field (different seasons) and on age of plants (nursery and mature) growing either in field or under green house conditions. However, best sterilization percentage was achieved in explants collected from young nursery raised plants in spring season.

Studies on seed sterilization have shown the maximum percentage on HgCl₂ (0.1-0.2%) for 10-20 min treatment (Nekrosova, 1964 in Prunus sps. and Mehra and Mehra, 1974 in almond) which is in agreement with the present studies, where 86.67% of seed sterilization was achieved on HgCl₂(0.2%) for 25 min. To strengthen the percentage sterilization score further, an improvement was obtained than first one where now 93.34% of seed sterilization was achieved by treating the pre-soaked seeds (in 1% NaOCl overnight) with a solution containing HgCl₂ (0.2g) and NaOCl (2ml) per 100 ml DDW, along with Tween-20 (0.05%) for 20 min. But these observations were not in line with the studies of earlier workers who used ethanol (70%) for 1 min. followed by NaOCl (0.5-20%) for 10-30 min. (Hisajima, 1982 in almond; Mante et al., 1989 in Prunus species; Pieterse, 1989 in apricot; Pooler and Scorza, 1995 in peach and Hokanson and Pooler, 2000 in cherry).

The current study revealed successful establishment of 2-3 cm long sterilized shoot tips of all the four cultivars of almond under reference on
MS (½) basal medium supplemented with sucrose (2%), Agar (0.7%) and BAP (1.5-2.5μM), with or without IBA (0.5μM), which corroborated to the observations of Ruguni and Verma (1982); Saeed (1998) in almond and Eldeen et al. (1998) in peach, who reported maximum rate of explant establishment on modified MS medium (1962) supplemented with BAP (2.2μM) and IBA (0.49μM). However, at the same time these findings contradict with observations made by earlier workers where maximum percentage of shoot establishment was reported on Knoops basal medium (Tabachnick and Kester, 1977 in almond) alone or in combination with BAP (4.4μM) and IBA (1μM) (Snir-I, 1982 in cherry) or on MS (½) medium fortified with BAP (3.0μM), NAA (0.05μM) and GA₃ (0.3μM) [Rodrigues et al., 1999 in GF₆₇₇ (peach x almond)] or on MS (½) medium supplemented with BAP (2.2μM) +GA₃ (12.99μM) (Chongshun et al., 1998 in Apricot.). Also the present studies are not in agreement with the observations of Anisley et al. (2000) in almond who have reported successful establishment on QL basal medium alone.

When IBA (0.5μM) was substituted by NAA (0.5μM), percentage of explant establishment and axillary bud sprouting got drastically reduced and instead callusing of explants was noticed, such observations differ with the one's recorded earlier in Prunus lauroceras (Ponchia and Gardiman, 1993) and in GF₆₇₇ (peach x almond) (Rodrigues et al., 1999) where NAA (0.05-0.5μM) was found to be helpful in explant establishment. At higher concentrations of BAP (4.4μM), with or without IBA / NAA (0.3μM), explants produced callus instead of axillary bud sprouting, during establishment phase, which again differ with the studies of Norton and Norton (1998) in Prunus spp. and Zotto et al. (1997) in plum, where explant establishment was reported on BAP (4.4-6.6μM). In present studies highest rate (70%) of shoot establishment, followed by axillary bud sprouting was achieved in cv. Parbat on BAP (2.5μM) + IBA (0.5μM), followed by cv. Shalimar on BAP (1.5μM) + IBA (0.5μM), cv. Mukhdoom and cv. Waris on BAP (2.0μM) alone. This difference in explant
establishment and subsequent response of axillary bud growth in different cultivars of almond under similar culture conditions, seem to be cultivar specific and needs further investigation.

In vitro studies of *Prunus* shoot tip explants have been carried out on different media viz. MS (1962), Knoop's salt mixtures, (1865), Anderson (1978), WPM (1984), TK (1977), LS (1977), QL (1965) and AP (1986) in presence of either BAP alone or in combination with IBA or NAA or GA₃ by different workers (Mehra and Mehra, 1974; Tabachnick and Kester, 1977; Shafar *et al.* 1985; Pevalek, 1985; Kester *et al.*, 1986; Norton and Norton, 1986; Dradi and Biondi, 1991 and Bouza, 1997). The results reported by all workers vary from medium to medium and from species to species. In present studies on shoot tip culture of four cultivars of almond under study, three different media were used namely Modified MS (1962) [macro salts reduced to half conc.], QL (1977) and AP (1986) in presence of BAP (2.5 to 9.0μM) either alone or in combination with IBA/NAA (0.05 and 0.5μM). Studies revealed that all the four cultivars differ in their proliferation rate on different media under same concentrations of BAP or BAP and IBA/NAA combination. Cultivar Parbat and Waris showed maximum rate of proliferation on modified MS medium with BAP (4.5μM) alone, which is in agreement to the studies of Pruski *et al.* (1992) in choke cherry; Sharma *et al.* (1992) in 'colt'(*P. avium* x *P. pseudocerasus*); Perez *et al.* (2000) in apricot and Anisley *et al.* (2000) in almond. However, at the same time these studies are not in agreement with the observations of earlier workers who used, Anderson medium (Shafar, *et al.*, 1985 on *P. bessey*), TK medium (Kester *et al.* 1986 in almond) and LP medium (Bouza, 1997 in *P. tenella*).

The addition of IBA (0.5μM) to BAP fortified media decreased the shoot proliferation rate in almond which does not corroborate with the findings made on similar nature of plants like Almehdi *et al.* (1982) in peach; Miller *et al.* (1982) in peach; Snir-I (1982) in sweet cherry;
Hammerschlag et al. (1987) in peach; Oh et al. (1991) in cherry; Ambrozic et al. (1992) in plum; Zotto, et al. (1997) in plum; Gurel and Guelson (1998 a & b) in almond and Muna et al.(1999) in cherry, who reported shoot proliferation on BAP (4.4\mu M) and IBA (0.49\mu M) combination. In present studies cultivar Shalimar and Mukhdoom showed highest rate of proliferation on QL (1977) medium in presence of BAP (3.0 and 3.5\mu M respectively). Shoot proliferation rate decreased in both cultivars when IBA (0.5\mu M) was added to BAP fortified medium. These observations however, differ with the reports of Dradi and Biondi (1991) in black cherry and Anisley et al. (2000) in almond where maximum shoot proliferation was achieved on QL (1977) medium supplemented with BAP (3.0-3.3\mu M) and IBA (0.049\mu M) in combination. The decrease in proliferation percentage may be attributed to difference in either species or cultivar status.

In current studies AP (1986) medium proved equally as effective as QL (1977) medium, both with BAP (4.5\mu M) concentration in shoot proliferation percentage for cv. Mukhdoom (40 and 44.44\% respectively) which is in contradiction to the findings of Anisley et al. (2000) in almond, where BAP(3.3\mu M) and IBA (0.05\mu M) combination produced maximum number of adventitious shoots per explant. Reports have shown that addition of NAA (0.05 to 5.3\mu M) to BAP fortified medium did increase the shoot proliferation rate in different Prunus sps. [Rugini and Verma, 1982 in almond; Rajit et al., 1988 in cherry; Ambrozic, et al., 1992 in plum; Ponchia and Gardiman, 1993 in P laurocerasus; Goudarzi et al., 1997 in cherry; Bizhu et al. 1998 in P. salicina; Shibli et al., 1999 in almond and Rodrigues et al., 1999 in GF$_677$ (peach x almond)]. In present studies substitution of IBA (0.05 and 0.5\mu M) by NAA (0.05 and 0.5\mu M) further decreased the percentage of shoot proliferation and adventitious shoot number produced per explant in all the four cultivars under study. These observations again did not agree with the findings of earlier workers who used different media with BAP (2.2-8.8\mu M), IBA/NAA (0.05-1.0\mu M) and GA$_3$ (0.29 to 0.6\mu M) [Ruzic et al., 1984 in GF$_677$ (peach x almond);
Maynard and Hall, 1985, in black cherry; Pevaleck, 1985 in wild cherry; Cerovic and Ruzic 1987, in sour cherry; Hammatt and Grant 1993 in cherry; Caboni et al. 1994 in almond; Bouza 1997 in *P. tenella* and Rodrigues et al., 1999 in GF677 (peach x almond).

Present studies have shown that increasing the BAP concentration beyond 4.5µM to 9.0µM either alone or in combination with IBA/NAA (0.05 and 0.5µM) markedly decreased the proliferation percentage, which differ with the findings of earlier workers who have reported maximum shoot proliferation on BAP (8.8 to 9.2µM) either alone or in combination with IBA (0.49µM) [Shafer et al. 1985 in *P. bessey*; Hammerschlage et al.1987 in peach; Sharma et al.1992 in *'colt'* (*P. avium* x *P. pseudocerasus*); Chatti 1994 in peach; Hasaballa et al. 1996 in apricot and Kumar et al. 1998 in plum]. Here the difference in response may be species specific.

Present study on 4 almond cultivars using three different media viz. Modified MS (1962), QL (1977) and AP (1986), exhibited difference in general growth and shoot proliferation rate under same phytohormonal concentrations which may be attributed to difference in mineral composition (nitrates) of different media and also the cultivar status. Shoot tip explants of cv. *Parbat* and cv. *Waris* showed normal growth and proliferation on MS (½) medium, but developed chlorosis, followed by death on QL (1977) and AP (1986) media. On the contrary explants of cv. *Shalimar* and cv. *Mukhdoom* showed normal growth and proliferation on QL (1977) medium, but explants of cv. *Shalimar* developed chlorosis and brittleness on MS (½) and AP (1986) media and on the other hand the explants of cv. *Mukhdoom* showed comparatively better growth on AP (1986) medium but perished on MS (½) medium. All these morphogenetic variations may be attributed due to genetic make up of different cultivars of the same species.
Further studies have shown that the microshoot elongation was achieved on MS (½) medium fortified with BAP (0.5µM) for cv. Parbat and cv. Waris and on QL (1977) medium with same BAP concentration for cv. Shalimar and cv. Mukhdoom. These observations showed close proximity to those of Tabachnick and Kester (1977) carried out in almond and Norton and Norton (1986) in *P. cerasefera*, who reported maximum microshoot elongation on same MS (1962) medium with BAP (0.44µM). However, these observations were contrary with those of Rugini and Verma (1982) in almond; Dradi and Biondi (1991) in Black cherry; and Sharma *et al.* (1992) in 'colt' (*P. avium* × *P. pseudocerasus*), where maximum shoot elongation was achieved on slightly higher concentration of BAP (0.88µM).

In present study MS (½) and QL (1977) basal media favoured the maximum (2 cm) shoot elongation of microshoots especially when groups (lumps) of shoots were subcultured, which may be attributed due to habituation of lumps on high BAP concentrated medium during proliferation phase.

The data scored for germination of seeds showed normal germination of chilled seeds (without seed coat) on MS(½) basal medium after 4 weeks. The published reports in this context from various workers (Ivanika and Pretova, 1986; Balla *et al.* 1996; Rizzo, *et al.* 1998) revealed that pretreatment of seeds with low temperature i.e.,1-5°C for 40 days was essential for normal germination, which otherwise resulted in poor germination, low vitality and crinkled leaves (Zagaja, 1962; Matveev *et al.*, 1997). Similar findings were also confirmed by present study, as kernels without chilling period of 40 days at 4°C failed to survive properly. Mehra and Mehra (1974) reported normal germination of almond kernels (without seed coat) on MS (½) basal medium similar to present study, but contradicts the normal in vitro development of seedlings in presence of GA₃ in cherry (Han-Lixing *et al.* 1999) or BAP and GA₃ in peach (Rizzo *et al.*, 1998). Further, studies on germination aspect revealed that the chilled kernels with intact seed coat (Testa) failed to germinate even after 8 weeks.
on MS (½) basal medium. This may be attributed to the presence of some germination inhibitors in seed coat. (Mehra and Mehra, 1974).

Studies on shoot regeneration revealed that there is maximum percentage of direct shoots regeneration possible from internodal segments of in vitro born seedlings cultured in presence of NAA (10μM) alone. However, addition of BAP/Kn (5.0 and 7.5μM) decreased the regeneration percentage, but on the other hand initiated shoot bud induction in internodal segments from nursery plantlets. Substitution of NAA (10μM) by IBA alone (10μM) further decreased the regeneration percentage in in vitro born explants, but again in combination with BAP/Kn (5.0 and 7.5μM) initiated shoot bud induction in internodal segments from mature trees. These observations suggest that the juvenile explants from in vitro born seedlings may contain some threshold level of cytokinin, which with exogenous supply of auxin (NAA or IBA) interacts and results in shoot bud induction. As maturity approaches in the explants it seems that the cytokinin level decreases drastically and the explants may have to depend upon exogenous supply of cytokinin (BAP/Kn) in addition to auxin (NAA/IBA) for shoot differentiation.

Hypocotyl segments from in vitro germinated seedlings also differentiated directly into adventitious shoots on MS (½) medium supplemented with BAP (2.5, 5.0 and 7.5μM) in combination with NAA (10μM) or IBA / 2,4-D (5 and 10μM). These observations proved contrary to those studies of Mehra and Mehra (1974) in hard shelled almond who reported indirect regeneration on MS (½) medium supplied with NAA (5μM) and Kn (1.0μM).

The induction of callus from root segments of in vitro born seedlings was reported by Mehra and Mehra (1974) on MS (½) medium with NAA (2-4μM) and CH (1g/l). Differentiation in this callus was observed on same medium enriched with NAA (5μM) and CH (1g/l). Present report shows 70 percent of root explants produced callus on BAP (5μM), NAA/IBA (10μM)
and L-glutamine (500mg/l) enriched medium. BAP and NAA combination produced non-regenerative callus, while BAP and IBA combination produced 10% regenerative callus upon subculturing on same medium, without glutamine. These observations differ with the studies reported by Dauart (1980), who recorded complete regeneration of *P. dawyckensis* and *P. canescens* plantlets from root callus on MS medium with BA (0.4μM) and GA₃ (0.037μM). Present observations also differ with those of Gutierrez *et al.* (1997) who recorded maximum plantlet regeneration from transgenic root calli of 'colt' (*P. avium x P. pseudocerasus*) on MS medium with BAP (4.4μM) and IBA (5.3μM).

The difference in in vitro regeneration of juvenile and mature plant material of almonds has been reported by Mehra and Mehra (1974) and Tabachnick and Kester (1977). A similar type of response was also observed in current studies on cotyledon culture of almonds. Immature cotyledons of almonds, 100-120 days after pollination (DAP), when cultured on MS (½) medium in presence of BAP (0-20μM) and IBA/NAA (5 and 10μM) differentiated into shoots and roots. Shoot regeneration percentage of 33.33 with highest adventitious shoot number of 7 produced per explant was achieved on BAP (20.0μM) in combination with IBA (10μM), which is not in accordance with the findings of Anisley *et al.* (2001 b) in almond, who reported 80 percent of regeneration on TDZ (20μM). In present findings shoot regeneration was also observed on BAP (5 and 10μM) in combination with IBA (2.5, 5 and 10.0μM). These observations are again not in conformity with those of Lane and Cassio (1986) in apricot and cherry; Pieterse (1989) in apricot and Schemidt and Ketzel (1993) in cherry, who reported shoot regeneration on BAP (4.4 and 8.8 μM) in combination with either 2,4-D (1.0μM) or IAA (5.7μM). In present study 80 and 100 percent of root regeneration from immature cotyledons was observed on IBA (5 and 10μM respectively), or 75 and 83.33% of root differentiation on NAA (5&10μM respectively) which is in close agreement with the observations of Mehra and Mehra (1974) in
almond, who reported 78% root differentiation on NAA (5\(\mu\)M) and Pieterse (1989) in apricot, on BAP (4.4\(\mu\)M) in combination with IBA (0.49\(\mu\)M). Shoot differentiation was also observed on BAP (5 and 10\(\mu\)M) in combination with NAA (5\(\mu\)M) in present study on immature cotyledons. In case of the mature cotyledons regeneration potential was seen to be very low or almost absent. Schemidit and Kardel (1992) and (1993) reported 40 percent shoot regeneration on BAP (4.4-8.8\(\mu\)M) and IAA (5.7\(\mu\)M) in cherry; Antonelli (1992) reported shoot differentiation on BAP (0, 2.2, 4.4\(\mu\)M) and NAA (0.53\(\mu\)M) in almond; Schmidt et al. (1996) reported 20 percent shoot regeneration on BAP (8.8\(\mu\)M) and IAA (5.7\(\mu\)M) in cherry.

In current studies on mature almond cotyledons, shoot regeneration with varying percentage was achieved on BAP (5.0 and 7.5\(\mu\)M) and IBA (2.5 and 5.0\(\mu\)M) combination. These observations were found not in agreement with those of Mante et al. (1989) in plum; Pooler and Scorza (1995) in peach; and Goeffreda et al. (1995) in apricot who reported shoot regeneration on TDZ (5-12.5\(\mu\)M) and IBA (2.5 and 5.0\(\mu\)M) combination. Very low percentage of shoot regeneration was also observed on BAP (2.5 and 5.0\(\mu\)M) or only on NAA (10\(\mu\)M), which testified the observations of Nedelcheva (1998) in apricot, who reported shoot regeneration on BAP (4.4\(\mu\)M) in combination with NAA (0.53\(\mu\)M).

Mehra and Mehra (1974) reported 8% shoot regeneration in mature cotyledons of almond on MS (\(\frac{1}{2}\)) medium with NAA (5\(\mu\)M) and Kn (1.0\(\mu\)M) and Nedelcheva (1998) on the other hand reported shoot regeneration on 2,4-D (4.5\(\mu\)M) and Kn (0.46\(\mu\)M) in mature apricot cotyledons. In our studies, 10-20% shoot regeneration from mature cotyledon explants was also achieved on NAA (2.5\(\mu\)M) and Kn (1.0, 5.0 and 10.0 \(\mu\)M). Further the dark incubation for 7 days increased the percentage regeneration potential, which is in accordance with the studies of Mante et al. (1988) in plum; Pooler and Scorza (1995) in peach and Anisley et al. (2001 b) in almond.
In leaf callus of almond Mehra and Mehra (1974) reported 10 percent shoot regeneration on NAA (5μM), Kn (1μM) and CH (1g/l) augmented MS (1962) medium while Hammerschlag (1988) reported only callus formation from leaf segments of peach on BAP (4.4μM) and NAA (0.27μM) combination. Likewise Maija et al. (1997) also reported only callus formation from leaf segments of almond on BAP (4.4μM) and GA3 (10.0μM) combination. Similar observations were also recorded by a number of other workers viz. Hammatt and Grant (1998) in P.serotina on WPM with TDZ (4.4 to 22.2μM) and NAA (0.5μM) combination and Declerk and Korban (1992) in peach on MS medium with TDZ (8-13μM) and Stabavitamins. Present findings on leaf discs of almond also exhibited similar results and calli of different degrees and nature (nodular, friable) was produced on BAP and IBA/NAA/2,4-D combination. But all these calli failed to differentiate into shoots or roots upon subculturing either on same medium, or on MS (½) basal medium or on MS (½) medium with BAP (4.4μM) and NAA (0.27μM) combination. These observations are not in agreement with those of Rugini and Verma (1982) in almond who reported indirect shoot differentiation from leaf calli on MS (½) medium with NAA, Zeatin and Casamino vitamins; Ochatt et al. (1987) in colt (P. avium x P. pseudocerasus) on BAP (0.44μM), IBA (0.67μM), NAA (0.53μM) and CH (500mg/l) and Hammerschlag (1988) in peach on BAP (2.2μM) and NAA (0.27μM) combination. Published reports of Maija et al. (1997) revealed that 80-90 percent regeneration was obtained from leaf segments of almond on BAP (4.4μM) and NAA (2.5μM) combination. Present findings again do not agree with the studies carried out by various workers where shoot regeneration from leaf callus was reported on TDZ in combination with either BAP or IBA (Nowak and Miczynski, 1996 in plum; Miguel et al., 1996 in almond and Anisley et al., 2000 in almond).

Present studies on embryo culture have shown that the production of adventitious shoots occur either directly or indirectly from embryonal axes of almonds. Direct regeneration of adventitious shoots from embryos of
other *Prunus* species like apricot (Pieterse, 1989; Esitken *et al.* 1999) and Peach (Schmidt and Ketzel, 1993) has been reported at BAP (4.4-8.8μM) and IAA (0.57-5.7μM) or 2,4-D (10μM). In current studies 62.22% of adventitious shoot regeneration was achieved at lower levels of BAP (2.5μM) and IBA (1.0μM) combination. However the inclusion of GA$_3$ (0.5μM) in regeneration media significantly reduced the number and frequency of adventitious shoot regeneration, which is in consonance with the studies of Rizzo *et al.* (1998) on peach and Han-lixing *et al.* (1999) on cherry, where GA$_3$ induced normal development of embryos instead of adventitious shoot regeneration. The current study on embryo culture also reveals the production of adventitious shoots indirectly from immature embryo callus, with maximum efficiency on BA (7.5 and 10μM) in combination with IBA (2.5 and 5.0μM). Such findings contradict the results of earlier workers who reported indirect shoot regeneration from embryogenic calli by using TDZ/BAP/Kn (0.44, 4.4 and 20μM) in combination with 2,4-D (1 and 4.5) or NAA (1.35μM) [Goeffreda *et al.*, 1995 in apricot; Pieterse, 1989 in apricot; Schneider *et al.*, 1992 in peach; and Tang *et al.*, 2000 in cherry].

Successful production of roots from shoots in current studies was noticed in all the four cultivars of almond. Rooting levels of upto 60% were achieved in cv. *Parbat*, *Shalimar* and *Mukhdoom* on IBA (2.5μM) through long dip method for 4 weeks. Both root initiation as well as elongation were achieved on the same medium. These results were in close conformity with those of Mante *et al.*(1989) in peach where 50-70% rooting was achieved on IBA (2.5-5.0μM) without subculturing on MS ( ½ ) basal medium for root elongation.

Highest percentage (85) of root initiation was achieved in all the four cultivars, when IBA concentration was increased to 3.0μM, followed by their elongation on hormone free MS ( ½ ) basal medium. These observations were partially in agreement with Cerovic and Ruzic (1987) in
sour cherry who reported only root initiation but not root elongation on IBA concentrated medium. Further, these results also showed close similarity with the observations of Bouza (1997) on *P. tenella*, and Sharma *et al.* (1992) in colt (*P. avium* × *P. pseudocerasus*) who reported root initiation on IBA (2.5-5.0µM) followed by root elongation on MS (½) basal medium. Tabachnick and Kester reported 50% rooting in almond on IBA (4.9µM) augmented medium. Rooting percentage of 33-55 was achieved in hard shelled almond on MS (½) medium supplemented with IBA (5.0-9.8µM) (Ochatt, 1980; Rugini and Verma, 1982; Rugini and Monastra, 1991). In present studies 30% rooting was achieved only in cv. *Parbat* on IBA (5.0µM), while the remaining cv's. viz. *Shalimar*, *Muckhdoom* and *Waris* failed to induce roots on IBA (5.0µM), which confirms the studies of Hisajima *et al.* (1982) in hard shelled almond, but contradicts the reports of Hasaballa (1996) and Goffreda *et al.* (1995) in apricot, Shibili *et al.* (1996) in almond and Dradi and Biondi (1991) in *P. serotina*.

In present studies no rooting was observed on higher IBA concentration (10µM) in all the four cultivars. The findings of Caboni and Damaino (1994) in contrast to present findings revealed 95% rooting in almonds on BN (½) medium supplemented with either IBA or IAA (10µM) in light.

Rooting percentage of 30-55 was reported in hard-shelled almond on NAA (4.9-10.8µM) by Tabachnik and Kester (1977), Rugini and Verma (1982) and Rugini and Monastra (1991). All these reports contradict present observations carried out on all the four paper shelled cultivars of almond, where zero percent rooting was achieved on NAA (2.5-10.0µM) fortified medium. This might be due to difference in gentic make up of cultivars in response to exogenous supply of hormone. It has also been reported earlier that excluding light from root zone by overlaying the media surface with black polycarbonate granules and painting the lower part outside of the
culture tubes improved rooting in almond. (Rugini et al. 1988, 1993). Similar observation was noticed in our studies in which a similar environment was provided by wrapping the culture tubes with black paper up to medium level and to which shoots responded positively. Without dark covering rooting was completely found absent.

For fruit trees it has been proposed that root induction occurs within few hours of auxin application and that prolonged application may not be necessary (Collet, 1988). Therefore, a suitable approach may be a pulse (or acute) application, whereby an auxin is applied at a high concentration over a short period of time. In our studies two such pulse methods (short dip and quick dip) were tested. Induction of shoots in water agar containing a high concentration of IBA (0.2g/l or 1.0mM) for 18 hours (short dip method) yielded significantly better response than quick dip method with IBA (1.0g/l or 5.0mM) for 5 min. in cv. Parbat and cv. Shalimar where 90 and 70 percent root regeneration was achieved upon subculturing on MS (½ ) basal medium after 6 weeks. While cv. Waris and cv. Mukhdoom totally failed in rooting response through such methods. Both these approaches viz. quick dip and short term incubation method were in conformity to the studies of Harbage et al. (1998) and De Klerk et al. (1997) in Malus spp., Popov et al. (1976) in sour cherry, Miguel and Oliveira (1999); Rugini and Monastra (1991) and Anisley et al. (2001 b) in almond where varying percentage of rooting was recorded by using IBA (0.1-1.0g/l). Further, out of the two auxins tested IBA was found to be a potent rooting hormone as compared to NAA in almond microshoots.

From the ongoing discussion of current work on four thin shelled indigenous cultivars of almond, it is evident that all the explants except leaf discs from all the cultivars showed in vitro regeneration potential in presence of plant growth regulators. The regeneration potential not only varied among these cultivars but also with hard shelled almond and other Prunus species under same or different phytohormonal combinations, which
is a clear indication of genetic behaviour of these plants to exogenous supply of hormones. Further highest regeneration potential was found to be in immature explants than their mature counterparts, which is an indicator of aging, as plant matures it fails to respond the external stimulus (hormones).

In the current study complete regeneration of plantlets from the shoot tips of all the four cultivars have maintained the clonal integrity, an essential requirement in plant breeding of such a type of extremely heterozygous (self incompatible) plant. Further indirect regeneration from different explants of these cultivars usher new doors for researchers to raise transgenics in terms of disease resistance, high yield and too late blooming.