Chapter 5

DISCUSSION
DISCUSSION

Results given in the preceding section raised certain points deserving discussion. The present findings in respect of Mangifera indica varieties, viz., Ambalavi, Dashehari, Romani and Chausa are considered in this respect.

In all the above-mentioned 4 mango varieties of M. indica, the response to nucellar embryogenesis was greatly influenced by the maturity of the nucellus explants as governed by the developmental stages of fruits. In all the cases, the maximum per cent response and frequency of embryo formation was evoked better in explants taken from younger than older fruits. The response of nucellus to embryogenesis finished after a particular maturity of fruits. Similarly, in mango variety Carabao, high frequency somatic embryogenesis has been obtained in nucellar explants taken from smaller than bigger fruits (Patena et al., 2002).

That the metabolic status of explants greatly influences its response to growth substances present in the medium as also their preferential requirement for growth hormones and nutrients is well documented in the literature. For example, in Capsella embryo culture, nutritional and hormonal requirements of a globular embryo for its growth are different from those of a heart-shaped or torpedo-shaped embryo (Raghvan and Torrey, 1963, ’64), as has been noticed in case of responses shown by the nucellus tissue of different maturity, which have been decided by the age of fruit as well as maturity of zygotic embryo in the present study. Interaction of developing zygotic embryo with nucellus, influencing its potentiality for polyembryony or its inhibition are some correlated processes under the control of embryo maturation gene, where the role of ABA and formation of certain stress inducing proteins, also termed as late embryogenesis abundant (LEA) have been stimulated (Williamson et al., 1985). To illustrate the point, a correlation has been demonstrated between the presence of ABA and levels of cruciferin and napin m-RNA’s synthesized in excised embryo culture of Brassica napus, where their high levels coincided with the peak of developmental stage of an embryo, beyond which their levels declined and the maturity phase set in (DeLisle and Crouch, 1989). However, the situation differs in the present case, where the embryogenic response
of nucellus tissue has been evaluated or observed in the absence of zygotic embryo, which all the more make the response precisely correlated only with the ambient hormonal and nutrient conditions of the medium.

It is interesting that in Ambalavi, embryogenesis was induced in mature nucellus by cytokinin alone, while the young nucellus of all the 4 varieties required presence of an auxin for better efficacy of cytokinin. In this context, an analogy may be drawn with the freshly isolated somatic callus tissue of *Citrus grandis* and the one resulted after its habituation in long-term culture, which required different cytokinins for differentiation of regenerants, i.e., BAP along with NAA induced differentiation in the former case but not in the latter for which only zeatin with NAA has been effective (Chaturvedi and Mitra, 1975). A poorer response of mature nucellus for inducing embryogenesis may be attributed to the increased concentration of inhibitors present in the more developed fruits, similar to the condition obtained with maturing embryos and seeds. Thus, if the state of monoembryony is due to the presence of certain repressor/s (growth inhibitors) present in the nucellus of monoembryonic mango varieties, which inhibit nucellar polyembryony as has been demonstrated by grafting experiments with nucellus of monoembryonic and polyembryonic *Citrus* species (Esan and Murashige, 1972; Tisserat and Murashige, 1977; Litz and Yurgalevitch, 1997), then the concentration of such repressor/s or inhibitor/s is expected to be higher in an older than the younger fruit. That is why, the nucellus of mature fruits of Ambalavi not only required a higher concentration of cytokinin for induction of embryogenesis, but also its extent in terms of the number of nucellar embryos induced per explant, which was less than that of the nucellus of younger fruits. Being monoembryonic varieties, in Ambalavi, Dashehari and Chausa, concentration of ethylene present in an increasing order with the age of fruits could be anticipated as one of the factors for suppressing induction of nucellar embryogenesis, which has been demonstrated to be so, in case of nucellar embryogenesis in another mango variety Tommy Atkins (Litz and Yurgalevitch, 1997). Similarly, embryogenesis in nucellar embryo callus of young fruit origin of *Citrus sinensis* and *C. karna* and production of shoot buds in callus from nucellar embryos of their mature fruit could also be
attributed to the maturity of nucellus (Chaturvedi and Sharma, 1987, ‘88). However, for certain varieties of mango, the reverse has also been reported to be true (Litz, 1989).

Consistent with other genera, the monoembryonic and polyembryonic species or varieties, like, citrus, the polyembryonic mango varieties investigated in this laboratory, viz., Totapari Red Small, Bappakai, Goa have been reported to be more amenable to evoke in vitro nucellar embryogenesis than the monoembryonic ones, viz., Safeda and Langra (Chaturvedi, 2003). In the present study also, induction of nucellar embryogenesis in monoembryonic varieties, like, Ambalavi, Dashehari and Chausa was comparatively more difficult and sporadic than the polyembryonic one, viz., Romani. However, the amenability of in vitro embryogenesis in mango is also reported to be variety specific. Since some varieties have been reported not to respond to any of the several treatments tested for embryogenesis as the Red Itamaraca (Mathews and Litz, 1992).

The specific role of growth hormones, particularly of auxins is not very certain in evoking nucellar embryogenesis in mango, albeit their presence has been conducive to embryogenesis in general. However, it was observed that, while in Dashehari a combined presence of BAP and TDZ used along with auxins, like, 2,4-D and NAA were effective in inducing nucellar embryogenesis, the combined presence of 2iP and BAP with 2,4-D and NAA was effective for Romani. In a still other case, presence of only 2iP was sufficient to do so in nucellus taken from developed fruits of Ambalavi but required an additional presence of an auxin, NAA in case of nucellus from young fruits. Here also, the specific role played by 2,4-D in inducing nucellar embryogenesis in any of the 4 mango varieties was not clear. Whilst in the studies with several mango varieties investigated by the Litz’s Group, 2,4-D has been found to play a decisive role in evoking response of nucellar embryogenesis, where its presence has been essential during the initial phase of suspension culture of nucellar tissue (Litz et al., 1982, ‘84; Litz, 1984; DeWald et al., 1989a & b; Jana et al., 1994; Ara et al., 1998, 2000a & b; Thomas, 1999; Patena et al., 2002; Dominguez et al., 2004).

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Nevertheless in the present study, initial exposure of nucellar tissue to 2,4-D was found conducive to induce the process of embryogenesis, but it was conditional to its subsequent withdrawal after 40 days in case of Romani. Such a step also appeared to be decisive in the pulse treatment strategy in Romani and Chausa as well as in second approach of induction of nucellar embryogenesis in Dashehari, for prior activating the nucellar tissue to divide and proliferate. Such responses of nucellar tissue to 2,4-D in triggering embryogenesis in general has been reported in several other plant species (Reinert, 1959; Thomas and Street, 1970; Kohlenbach, 1977), where the tissues have been initially exposed to high concentration of 2,4-D, e.g., in Bamboos (Mehta et al., 1982; Rao et al., 1985; ElHasan and Debergh, 1987); Macalena cordata (Kohlenbach, 1959); sugarcane (Nadar et al., 1978); peach (RajBhansali, 1989); pomegranate (RajBhansali, 1990) and citrus (Tisserat and Murashige, 1977; Raj Bhansali and Arya, 1978). It is in conformity with the basic concept that sequential exposure of tissue initially to high auxin or 2,4-D, wherever it is effective, followed by its withdrawal triggers organogenic differentiation or somatic embryogenesis (Kohlenbach, 1977; Litz et al., 1984). However, the auxin concentration necessary for the induction of embryogenic state later hinders its defined development as the concentration rises. High auxin concentration results in disorganized embryogenic structures, which develop to organized embryos only after withdrawal of auxin (Kohlenbach, 1977).

In the present study, cytokinins were used along with auxins for induction of embryogenesis. Like many other plant species, in wild cherry, BAP and Kn have been used along with NAA for induction of embryogenesis (Garin et al., 1997). In a wide variety of species, 2,4-D and NAA, alone or in combination with cytokinins have been used in the induction and proliferation of somatic embryos (Attansov et al., 1984; Stuart and Strickland, 1984; Brown, 1988).

The precise mechanism of action of TDZ is not known; either it promotes growth due to cytokinin-like activity or accumulation of endogenous cytokinins (Capelle et al., 1983; Mok et al., 1982; Mok and Mok, 1985) or cytokinin as well as auxin-like properties (Khurana et al., 2005). Bates et al. (1992) reported induction of somatic embryogenesis in Fraxinus sp. on a medium containing high auxin: low
cytokinin, i.e., 10 μM 2,4-D with 1μM TDZ. However, it has been highly efficacious in stimulating somatic embryogenesis in several plant species including geranium and _Malus_ (Visser _et al._, 1992; Daigny _et al._, 1996), particularly effective in woody plant species (Khurana _et al._, 2005). In the present study, exposure of nucellus of Dashehari and Romani to TDZ along with 2,4-D and NAA for 7-10 days was sufficient to evoke embryogenesis.

Induction of embryo differentiation in nucellar tissue in all the 4 varieties occurred in a localized manner and was generally confined near the micropylar region of the ovular half. In most of the cases, only 1 - 2 or rarely 4 embryos differentiated at 1 or 2 locii, while rest of the embryos resulted from proliferation of earlier formed embryos under a non-synchronized process of differentiation but a continuous process where earlier formed embryos did not inhibit differentiation of new embryos. This phenomenon is common to most of the species investigated for somatic embryogenesis (Halperin and Wetherell, 1964; Chaturvedi, 1968; Schumann _et al._, 1995; Karkonen, 2000) as also caulogenesis (Chaturvedi and Mitra, 1974). Furthermore, the histological pattern of differentiation of nucellar embryos followed the same scheme as of other plant species, where first pockets of proembryogenic tissue were formed followed by differentiation of meristemoids which ultimately gave rise to embryos due to tissue differentiation within themselves (Bunning, 1952; Reinert, 1959, '64; Halperin and Wetherell, 1964; Reinert _et al._, 1977). Similarly, like somatic embryos formed in any other plant species, as also in mango any 2 nucellar embryos differentiated in the aforesaid fashion were identical, but on convertibility they produced plantlets with perfect characteristics of the particular variety, to which the nucellar embryos belonged.

Utilization of nucellar embryogenesis for continued production of nucellar plantlets entails sustained proliferation and maintenance of fully differentiated embryos in prolonged culture. Notwithstanding the requirement of particular cytokinin and auxin combination at a much higher concentrations in the induction phase than the maintenance phase, for all the 4 mango varieties, scion as well as rootstocks, agarified nutrient medium was used in both the phases. Whilst in studies by Litz’s Group for American mango varieties (DeWald _et al._, 1989a; Litz
et al., 1984, 1993, '95) as well as by certain other investigators in the country for Indian varieties (Ara et al., 1998, 2000) the initial phase comprised suspension culture of nucellar tissue, which has been later utilized for induction of nucellar embryos. The maintenance phase also has been sustained in such studies in the liquid medium.

A comparative assessment of the 2 strategies, the strategy in which suspension culture of nucellar tissue has been employed may prove disadvantageous in view of incidence of somaclonal variation. Occurrence of somaclonal variants has been reported in cultures of a number of mango varieties investigated (Litz et al., 1993, '95). Whereas in the present case, employment of semisolid medium eliminated the possibility of occurrence of somaclonal variation, which is of high significance when clonal multiplication is concerned.

In general, nutrient media for sustained proliferation of nucellar embryos varied from the one used for induction not only in respect of the concentration of particular cytokinin and auxin, but also the type of cytokinin and auxin required for producing optimum results. For example, in Dashehari, where TDZ and BAP along with 2,4-D and NAA were instrumental in induction of nucellar embryogenesis, sustained proliferation of embryos was obtained in a medium containing low concentrations of BAP and 2iP along with IAA. Also in Romani, where TDZ along with 2,4-D and NAA played a decisive role in induction of nucellar embryogenesis via the second approach after the manner of Dashehari, prolonged cultures of proliferating embryos were maintained in the medium containing 2iP, BAP and IAA. Such sequential and qualitative difference in the requirement of cytokinins and auxins during the induction phase of nucellar embryogenesis and maintenance phase have rarely been described by other workers. In addition to this aspect, the requirement of cytokinins and auxins further varied for proliferation of nucellar embryos in prolonged or long-term culture. The point is well exemplified in case of nucellar embryos of Romani, where 0.25 mg l⁻¹ each of BAP and 2iP and 0.5 mg l⁻¹ NAA effected embryo-to-embryo proliferation, but the rate of proliferation progressively declined after few subcultures. Lowering the concentrations of BAP and 2iP to 0.15 mg l⁻¹, whereas
substitution of 0.5 mg l\(^{-1}\) NAA by 0.5 mg l\(^{-1}\) IAA restored embryonal proliferation, which progressively increased to the optimum. Failing such precise changes in hormonal and nutritional composition of media for induction, short-term maintenance and prolonged or long-term culture of nucellar embryos, the amount of necrosed proembryogenic tissue along with young embryos continued to increase with the progressive subcultures, resulting in progressive decrease in number of available normal nucellar embryos per culture. It defeats the very purpose of having a stock of well-differentiated embryos, from which embryos could be taken from time to time for convertibility into plantlets. Like cytokinins, requirement of the 4 mango varieties for AdS also varied. Whilst Romani had the minimum requirement of 15 mg l\(^{-1}\) AdS for sustained maintenance of proliferating embryos, Dashehari and Chausa required a higher concentration of 25 mg l\(^{-1}\) AdS and the Ambalavi had the maximum requirement of 50 mg l\(^{-1}\) of AdS.

Besides proper ratio of cytokinin and auxin supplemented to the medium, inorganic salt composition also greatly influenced regenerant differentiation as had been exemplified by the use of different concentrations of CaCl\(_2\) in somatic embryogenesis of Romani. Lowering CaCl\(_2\) concentration increased the efficacy of BAP and 2iP in respect of enhancing embryo proliferation. A lower concentration of 100 mg l\(^{-1}\) CaCl\(_2\) promoted more proliferation than maturation and vice versa. Such a decisive role played by CaCl\(_2\) concentration in affecting cytokinin action is similar to that reported for caulogenesis in leaf explants of *Rosmarinus officinalis* (Misra and Chaturvedi, 1991, '93). However, at this stage of our understanding of regulation of plant growth hormones, it is not possible to interpret such results. In *R. officinalis* its total absence in the medium promoted caulogenesis by BAP and IAA as compared to that obtained even at its very low concentrations.

A major drawback in proliferating cultures of nucellar embryos in mango has been necrosis of embryogenic tissue and of young embryos, as also fasciation of embryos or embryos with other abnormalities, like, absence of proper plumular region or vitrification, which greatly reduced yield of normal embryos per culture as was in the case of Chausa. The situation can be compared, although to a lesser degree, with what has been reported for proliferating nucellar embryo cultures of
Totapari Red Small. It is well documented in literature that somatic embryogenesis can be suppressed by exogenously supplied ethylene (Tisserat and Murashige, 1977; Roustan et al., 1994) or its precursor, 1ACC (1-aminocyclopropane-1-carboxylic acid) (Songstad et al., 1988, '89; Vain et al., 1989). Attainment of normal embryogenesis with differentiation of well organized embryos during the process of their proliferation with the use of AVG and to some extent AgNO₃ substantiates the fact that ethylene induces abnormalities during the process of differentiation of regenerants in vitro as obtained in the present study in the form of fasciation of embryoids (Chi and Pua, 1989; Chi et al., 1991; Pua and Chi, 1993). The reported stimulation of somatic embryogenesis in M. indica var. Tutehau by reducing ethylene biosynthesis with AVG by Litz and Yurgalevitch (1997) and also control of fasciation and promotion of individual development of embryos of M. indica var. Totapari Red Small by the use of AVG by Chaturvedi et al. (2003) also support the aforesaid observations. However, it has been reported when a low concentration of AVG was used, which did not inhibit ethylene biosynthesis, somatic embryogenesis in Medicago sativa remained inhibited, pointing thereby that it is the concentration of available ethylene in the culture vessel, which directly affects somatic embryogenesis (Meijer and Brown, 1988).

The main difference in controlling fasciation and abnormalities in proliferating embryos of Chausa and the other mango varieties described above was that instead of AVG, 5 mg l⁻¹ ancymidol along with a high concentration of 500 mg l⁻¹ m-inositol was efficacious.

In general, other investigators who studied somatic embryogenesis in tropical fruit tree species have experienced difficulty in controlling somatic embryo development to full maturity and their germination (Wang and Janick, 1984). Ammirato (1983) stated that somatic embryogenesis and subsequent development of embryos and their germination, for many plant species can involve a change in culture conditions.

For further development of nucellar embryos of all the 4 mango varieties investigated, viz., Ambalavi, Dashehari, Romani and Chausa, the isolated embryos had to be processed in a medium different from the one, which supported their
sustained proliferation and development. First, it had to be in the liquid state; secondly, it mainly had a lower concentration of nitrogen; and third, it had a supplement of a growth inhibitor or retardant, particularly ABA along with a high osmoticum creating substances particularly PEG so as to create stress conditions simulating the situations obtained in nature during embryo and seed maturation.

In the present study, all the above conditions were fulfilled by incorporating 0.01 mg l\(^{-1}\) ABA and 100 mg l\(^{-1}\) PEG along with 0.1 mg l\(^{-1}\) IAA in a liquid medium having low nitrogen content. The same medium was equally effective for all the 4 varieties investigated. Depending on the length of incubation in the same medium which involved subculturing as well as the stage of embryo development to its full size and maturation in which the developed embryo showed radicle growth during 40 days of incubation, germination when plumule also become visible along with radicle growth during further 30 days and ultimately their conversion into plantlets during another 30 days of incubation, which involved subculture of germinated embryos in the same medium. In other mango varieties as well as in other plant species, maturation of somatic embryos has been dependent on the above mentioned conditions except that different substances were effective to create stress conditions. Increased stress conditions, which are generally created by employing PEG, D-sorbitol or D-mannitol used with or without ABA, have been found conducive to development and maturation of somatic embryos of various varieties of mango as well as of some other plant species (Attree et al., 1991; Pleigo-Alfaro et al., 1996a & b; Litz and Lavi, 1997; Walker and Parrot, 2001). PEG in combination with sucrose has been effective in *Hevea brasiliensis* (Etienne et al., 1993).

But the ABA plays a double or multiple roles in embryo maturation besides being acting as a hormone, synchronizing and shaping the normal development of embryos. In nature, ABA has a role to play in the metabolism of production of stress proteins of embryos by activating particular gene or genes responsible for production of such proteins (Walton, 1980; Bray and Beachy, 1985; Crouch et al., 1985; Williamson et al., 1985; Galau et al., 1986; Morris et al., 1990; Roberts et al., 1990).
Germination of matured embryos required presence of IAA, along with ABA and PEG, while BAP and GA suppressed it. This effect of BAP and GA on germination of processed mature embryos is contrary to that obtained in nature, where both these hormones have been found to promote seed germination (Copeland and McDonald, 2001). Also, GA has been reported to promote development as well as maturation of mango varieties by Ara et al. (2000b). The beneficial effect of BAP on somatic embryo germination has also been reported in different cultivars of mango (Litz, 1984; Jana et al., 1994; Laxmi et al., 1999).

In concurrence with the present observations, IBA have been reported to facilitate somatic embryo maturation and germination (conversion) in larch (Von Aderkas et al., 2001).

The role of NH\textsubscript{4}NO\textsubscript{3} in promoting somatic embryogenesis in vitro has long been discussed, starting from embryogenesis in carrot tissues (Steward, 1954; Reinert, 1962; Halperin and Wetherell, 1965; Halperin, 1964, 1995). However, no general rule could be formulated for all the plant species. But in plant growth, generally the CN ratio decides vegetative growth when N > C and reproductive phase when C > N. With the same analogy, reduction in nitrogen content of the medium mainly the reduced nitrogen was promotive to embryogenesis and enhanced percentage of convertibility under the influence of other growth hormones and other constituents of the medium.

Akin to such an aberrant behaviour of otherwise similar looking nucellar embryos of mango, nucellar embryos of citrus differentiated in vitro may either germinate to form plantlets or produce only proliferating cotyledon-like structures and these 2 types of behaviours are unpredictable on the basis of appearance of embryos. Unpredictability of otherwise uniformly looking embryos for germination and for germinated embryos to develop into plantlets, is the aspect that has not been discussed in the literature dealing with maturation and convertibility of nucellar embryos of mango in particular and other plant species in general, whereas if it could be unravelled, the production of quantified number per cultures could be assessed and production of plantlets from in vitro cultures to be regulated according to their demand in the trade. This phenomenon appears to be more
unique with mango than other plant species where the well-developed and mature embryos could mostly be predicted to produce plantlets on germination. Based on such an attribute of mature embryos, the concept of artificial seeds has been developed (Redenbaugh et al., 1986 '88; Slade et al., 1989; Ara et al., 1999) and even raising of man-made forests by sowing artificial seeds has been contemplated.

In *M. indica* var. Amrapali ca. 90% rooting has been reported with IBA in a 2-step procedure employing a pulse treatment of microshoots. However, the advantage of adventitious rooting of microshoots over nucellar plantlets in respect of transplant success and *ex vitro* growth have not been tested (Ara et al., 1998). However, in the present study 90% rooting was induced when a 3-step procedure employing pulse treatment of 24h of multiple auxins (IBA, NAA and IAA) with phloroglucinol at the 1st step and subsequent transfer of shoots to low concentration of IAA with phloroglucinol and after the induction of root primordia their culture in a very low concentration of auxin. There has been virtually no intervenal callusing through this procedure.

Plantlets of all the 4 varieties resulted from processed developed embryos appeared normal and healthy in all respects, except that root system was delicate and brittle and in general lacked in lateral roots. Plantlets were variously hardened before transplantation to the potting mixture. Besides *ex vitro* hardening, the plantlets were also hardened *in vitro*, albeit with the progress in incubation period, necrosis of leaves, shoot tips and even blackening of stem ensued, which was most prominent in Chausa followed by Romani, Dashehari and Ambalavi. Both the well founded procedures of hardening of *in vitro*-raised plantlets, one developed for *in vitro*-raised plantlets of *Dioscorea floribunda* (Chaturvedi, 1975) and the other developed for a very intractable to acclimatize plant species, namely, *Simmondsia chinensis* (Chaturvedi and Sharma, 1989) were followed with better results of transplant success in the second case.

The blackening of stem which led to the death of shoot tip and ultimately the whole plant may not be a pathological problem, but of functional anatomy, since it occurred under aseptic conditions also. Histological preparations revealed disintegration of tissue organization in the blackened portion of stem including
water and food conducting tissues (xylem and phloem) demarkated by normal histo-differentiation in green portion of stem below and above its blackened portion.

Notwithstanding the procedure of acclimatization followed, the plantlets generally developed necrosis of tips of shoots and leaves also showing inward curling, which was more pronounced in Romani followed by Dashehari, Chausa and Ambalavi, and in due course of time, ranging from 2 to 5 months, the plantlets died. Litz’s Group also faced a great difficulty in getting transplant success of in vitro-raised plants of mango varieties. DeWald et al. (1989) presumed the poor transplant success to be due to non-functional or not so well functional root system of the nucellar plantlets. They reported alleviation of this problem through foliar feeding with inorganic salts. However, in the present study, foliar feeding with major and minor inorganic salts as present in modified Knop solution or with hormone mixture comprising IAA, Kn as also GA used separately and in combination with auxins and cytokinins did not make any appreciable difference with reference to untreated control plants.

The theory of non-functional root system of in vitro-raised mango plantlets, was further examined by adventitious rooting of healthy shoots excised from nucellar plantlets, which were expected to be functional since they appeared to be healthy and not so brittle, but such rooted shoots of all the 4 mango varieties did not perform well compared to their counterparts i.e., intact nucellar plantlets. It may thus be inferred that the cause of non-survival of plantlets ex vitro might be due to some other reasons. On this score, the various potting mixtures were tested to achieve better transplant success and ex vitro survival of in vitro-raised plantlets. Of the various potting mixtures used, which comprised garden soil taken from the rhizosphere of mango tree, Soilrite, Soilrite plus soil, Soilrite plus, Vermiculite, etc., it did not make any appreciable difference regarding survival of plantlets, except that Soilrite was slightly better than others. Drenching of potting mixture with inorganic salt solution (Modified half-strength Knop’s solution) once in a week was also of no avail. Similarly, inoculation of root system of plantlets with VAM was futile.
Longevity of survival of in vitro-raised plantlets using the acclimatization procedures of Chaturvedi and Sharma (1989) and employment of Soilrite as the potting mixture gave better results not only in respect of per cent transplant success, but also the duration of survival of plantlets ex vitro, which was further improved when plantlets were transplanted just at the onset of Rainy Season so as to simulate the natural season of germination of stones of mango, while the plantlets were kept in the net house. The maximum survival of plantlets differed with different mango varieties being maximum in Ambalavi for 6 months followed by Dashehari and Chausa for 5 months and minimum for Romani, i.e., 4 months. The plantlets after transplantation under ex vitro conditions did grow and in certain cases 2-3 pairs of new leaves and an increase of stem by ca. 2 cm in length took place before the setting in of necrosis. It is intriguing that if the failure of plantlets to survive is due to the non-functional root system than how they survived for such a long period as 6 months and how the new growth took place? It may not be wrong to conclude that the cause of mortality of in vitro-raised plantlets may be due to certain other factors than simply the non-functional root system. But the fact remains that, there is no mention of survival of thousands of in vitro-raised mango plants produced world over by so many workers during the past 20 years beyond transplantation not to mention their further development to maturity and coming to the fruiting stage. In the present study also, in all about thousand plantlets of all the 4 varieties combined were transplanted for ex vitro growth, but none survived beyond 6 months. This global problem of non-survival of in vitro-raised plantlets under ex vitro conditions is the most outstanding problem that needs solution with a multidisciplinary and concerted approach.