Chapter VII

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The present investigation on *in vitro* propagation, quantitative estimation of alkaloids and steroids and analysis of variants in *Withania somnifera* is discussed below.

I. *In vitro* propagation

Effect of age of explant

The effect of season, age of the explants and the effect of various growth hormones on induction of sprouting was studied simultaneously. The sterilized nodal explants were inoculated on MS medium supplemented with vitamins and hormones alone or in combination of two. Explants from non flowering plants showed 85% sprouting within 10 days. The part of the explants which was in contact with medium swelled as an activity of meristematic activity. This was accompanied by abscission of subtending leaf. The portion of the explants above the medium did not show any callusing. Bud growth from nodal explants of the mature flowering plants was negligible; most of the axillary buds turned light brown and died. However only 10 to 20 % apical tips and first three nodal explants from matured flowering plants showed initiation of single sprout after 20 to 25 days of incubation on initiation medium. Continued incubation did not show any improvement in the formation of shoots.
However, the explants either died or the entire explants formed non fragile callus. The newly sprouted branches from non flowering plants were more responsive than those from mature, flowering plant of *Withania somnifera*.

**Effect of position of explant**

The axillary buds of the newly sprouted branches, which were nearer to the apical bud, were more responsive. After about three or four axillary bud the stems becomes woody and such buds showed no response in culture. In the preliminary experiments, effect of cytokinins BAP, KIN, 2iP alone or in combination were tested. Maximum (70-80%) number of bud break and initiation of shoot was reported in BAP alone (1.0 to 2.0 mg/l), about one to two shoots were developed in the cultures contained BAP and followed by KIN. With 2iP there is no any initiation of shoot has been reported. The combination of BAP and KIN shows 40 to 45% of initiation was found to the formation of only one shoot with the formation of callus. The maximum length of shoot was observed (one to two cm) in the medium containing BAP alone in comparison to the medium supplemented with BAP with KN. Since the preliminary experiments indicated the synergistic effect of BAP and KIN and on sprouting of shoot further experiments were conducted to evaluate the optimum concentration of cytokinins. Only the combination of lower concentration
of BAP and KIN produced best results while higher concentration of BAP favoured less, short and weak shoot with the formation of more callusing from the explants base.

For the selection of suitable explants and establishing successful plant tissue culture system, it is essential to have knowledge on natural propagation system of plants. The time of the year when explants are collected from stock plants may have an influence on auxiliary shoot out growth. The best period of the year for initiating shoot bud culture from nodal explants of *Withania somnifera* was noted to be May-June. During this period the plant exhibited maximum bud break percentage, while contamination was noted to be very low or no contamination in March-April.

**Seasonal changes in plants and response of plants to contamination:**

During *in vitro* propagation of some plants certain types of slow growing microbial contaminants persist even after initial surface sterilization of explants. Such contaminants may persist for many generations without being noticed and cause reduction in vigour or chlorosis in propagated plantlets (Knauss and Miller, 1978). The time of the year that the explants are collected from stock plants may have an influence on axillary shoot outgrowth. The culture initiated from nodal explants of *Withania somnifera* exhibited maximum contamination (90%) during July-August.
This indicates that the bud break response and contamination of the cultures varied depending on the season of the explants isolation from the mother plant. Fluctuation in the environmental factors in different seasons had a definite effect on shoot bud differentiation from explanted nodal segments in *Withania somnifera* and *Adhatoda vasica* as similarly observed in other medicinal herbs including *Ocimum* species (Ahuja *et al.*, 1982; Patnaik and Chand, 1996), *Tridex procumbens* (Sahoo and Chand, 1998) and *Solanum surattense* (Swamy *et al.*, 2004).

Various workers investigated the problem of microbial contamination of culture especially serious when the tissue was derived from field grown material. In this respect, the behavior of explant in culture may be decided critically by the season and growth stage of the parent plant. Hohtola (1988) suggested that during winter months decreased contamination might be due to the better resistance of the tissue against microbes during active period of growth and/or susceptibility of microbes to the decontaminants. Anaz and Vijay Kumar (1997) attributed the high level of contamination during rainy month to the amount of inoculums present in the environment due to favorable condition. To ensure complete conditions of the explants it is essential to remove dirt and debris from the plant tissue. To improve wetting of the tissue surface, a detergent or alcohol wash often precedes treatment with steriliants. Ethanol partially removes hydrophobic waxes and resins, which protect
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microorganisms from contact with aqueous sterilants (Kunneman and Faaij-Groenen, 1988).

Effects of explants

Shoot tips and nodal segments were used as explants at establishment stage. Of these two, nodal segments responded better than shoot tips. Nodal bud cultures were found to be an efficient means of clonal multiplication of plants by several workers too. Plantlets were successfully produced by culturing shoot tips with a couple of primordial of Lupinus and Tropaeolum (Ball, 1946). Direct in vitro clonal propagation for nodal explants had been achieved in Euphorbia lathyris and Euphorbia plepus (Tideman and Hawker, 1982), Euphorbia fulgens (Zhang et al., 1987), Jatropha curcas (Sardana, 1998), Wedelia chinensis (Emmanuel et al., 2000), Ocimum sanctum (Shahzad and Siddiqui, 2000), Hyptis suaveolens (Britto et al., 2001), Jatropha curcas (Rajore et al., 2002), Tinospora cordifolia (Kumar et al., 2003) Withania somnifera (Vadawale et al., 2004), Centella asiatica (George et al., 2004; Shashikala et al., 2005), Tabebuia serratifolia (Nery et al., 2008), Elaeagnus angustifolia (Zeng et al., 2009), and Ceropogia thwaitesii (Muthukrishnan et al., 2012). There were also records of studies that was done on various other plants which used different other plant parts to induce callus such as leaves, nodes and buds (Mungole et al., 2009). In vitro clonal multiplication of Kaempferia galanga through rhizome buds
was reported (Vincent et al., 1992; Geetha et al., 1997; Lakshmi and Mythili, 2003). A few reports available described the *K. galanga* micropropagation using rhizome pieces (Shirin et al., 2000, Swapna et al., 2004). There are a number of reports regarding *in vitro* regeneration of *Withania somnifera* L. Dunal by using various explants such as shoot tips (Sen and Sharma, 1991), nodal segments (Tiwari and Singh, 1991, Kulkarni et al., 2000), axillary meristems (Roja et al., 1991), axillary shoots and hypocotyls and root segments (Rani and Grover, 1999).

**Effects of used media**

The earliest nutrient media used for growing plant tissues in vitro were based on the nutrient formulations for whole plants. First significant attempt of isolated plant cells on artificial nutrient medium was done by Haberlandt (1902). Subsequently various culture media were reported to obtain cultured cells in diverse plant explants of several taxa by the work of Hildebrandt et al. (1946), Nitsch (1951), Reinert and White (1956), Murashige and Skoog (1962), White (1963), Gamborg et al. (1968), Schenck and Hidebrandt (1972). Among several media employed in tissue culture studies, the most suited formulation was Murashige and Skoog (MS medium) which has well balanced micro- and macronutrients besides having a highest concentration of nitrogen compared to other
media. MS formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micronutrients, a carbon source, reduced nitrogen, vitamin B and growth regulators (Gamborg et al., 1976). The MS salt formulation is now the most widely used nutrient medium in plant tissue culture. In *Withania somnifera* the formulation of Murashige and Skoog’s, 1962 basal medium was found more suitable as against other media.

**Effects of growth regulators**

Growth regulator concentration in the culture medium is critical to control the growth and morphogenesis. Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promoted abundant cell proliferation with the proliferation of callus. On the other hand, low auxin and high cytokinin concentrations in the medium resulted in the induction of shoot morphogenesis. Auxins alone or with a very low concentration of cytokinin were found important in the induction of root primordial. The role of cytokinins in shoot organogenesis is well established (Evans *et al.*, 1983). Different plants were expected to respond differently to various cytokinins and auxins and this may be partly because of their endogenous hormonal levels. Supply of hormones in a proper sequence was important to achieve a particular
response. It is well established that proper ratio of cytokinin and auxin is necessary for morphogenesis leading the formation of complete plantlets (George and Sherrington, 1984). Auxins could regulate and influence diverse response on a whole plant level, such as tropism, apical dominance and root initiation and responses on cellular level such as cell enlargement, division and differentiation (Hagen and Guilfoyle, 2002). It was clearly documented that generally, high concentration of auxins and low cytokinins in the medium promote abundant cell proliferation with the formation of callus (Shah et al., 2003). Cytokinins act at the cellular level by inducing the expression of some genes, mitotic promotion and chloroplast development but also on the organ level by releasing buds from apical dominance or by inhibiting root growth (Riefler et al., 2006).

Thus auxin and cytokinins interact in the control of many central developmental processes, particularly in apical dominance and root and shoot development.

There are reports that using MS medium supplemented with 3.0 mg/l 2,4-D and 0.5 mg/l BAP induced callus (Paramageetham, 2000). He also showed that best results for callus formation in Bacopa monnieri (L.) Penn. was obtained in the leaf explants on MS supplemented with 0.5 mg/l 2, 4 -D. The different concentrations of BAP in MS medium influenced the shoot number per explant, shoot length, node number per shoot and leaves number per shoot. Callus showed a different response
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according to the growth regulators used. Kinetin seems to be essential for further multiplication and growth of shoots. When explants with shoots and shoot buds were subcultured on the same medium, shoot buds did not develop into shoots, but when subcultured on medium containing kinetin, shoot development was seen. In cultures raised from nodal explants of Withania somnifera maximum numbers of shoots were produced on MS medium supplemented with 0.1 mg/l BAP. These explants developed greater than two shoots per node while, in all other concentrations of NAA and kinetin developed either two or less than two shoots per explant. BAP, NAA and kinetin at a concentration range 0.1-1.0 mg/l were tested for assessing the optimum concentrations of the cytokinins for early sprouting and maximum proliferation of axillary shoots. BAP was found to be more effective than NAA and kinetin on the proliferation and development of Withania somnifera shoots. Superiority of BAP over other cytokinin in producing in vitro shoots had also been confirmed in other plants. The explants show shoot initiation after 7-10 days. The medium with growth regulator BAP produced greater number of shoots than the basal medium as also been reported in Zingiber officinale (Balachandran et al., 1990); Hoppea odorata (Scott et al., 1995); Murraya koenigii (Rajendra and D’Souza 1998); Celastraus paniculata (Nair and Seeni, 2001); Vitex negundo (Chandramu et al., 2003); Adhatoda vasica (Rahman et al., 2004); Arbus precatorius (Biswas et al., 2007);
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*Kaempferia galangal* (Jinu and Aravindan, 2008); *Hymenocallis littoralis* (Aftab et al., 2008) and *Ricinus communis* (Nahar and Borna, 2012). The synergistic effect of higher concentration of cytokinin with lower concentration of auxin induced better shoot formation was reported by various workers in different plant species (Mishra et al., 2004 and Vidya et al., 2005). However, some other hormones have also been added by some of the workers along with BAP for some other plants like *Solanum viarum* (Tejavathi and Bhuvana, 1996); *Alpinia galangal* (Anand and Hariharan, 1997); *Passiflora caerulea* (Jasrai et al., 1999); *Vitex negundo* (Thiruvengadan and Jayabalain, 2000, Sikdar et al., 2003); *Hypericum patulum*, (Baruah et al., 2001); *Cajanus cajan* (Vijayakumari et al., 2001); *Withania somnifera* (Bansal and Baghel, 2010); *Ricinus communis* (Alam et al., 2010); *Cicer arietinum* (Riazuddin et al., 2012). In the present study, when explants were cultured in MS medium supplemented with various concentrations of kinetin, single healthy shoot was produced in the media composition. This is in accordance with the results as reported earlier by Neeti and Kothari (2005) who worked on *Eclipta alba*. In the present investigation, higher concentration of cytokinin reduced the shoot number as well as shoot length. A similar response was observed in *Centella asiatica* (Nath and Buragohain, 2003) and *Terminalia chebula*. (Shyamkumar et al., 2004). Of the two cytokinins used for multiple shoot induction in the present
study, BAP induced more number of multiple shoots compared to kinetin. The superiority of BAP over kinetin in inducing large number of multiple shoots has been reported in several plants by several workers; *Gymnema sylvestre* (Komalavalli and Rao, 2000); *Cunila galioides* (Fracaro and Echeverigaray, 2001); *Stevia rebaudiana* (Mousmi, 2008) and *Acacia catechu* (Jain et al., 2009). Rooting of *in vitro* derived shoots of *Withania somnifera* and *Adhatoda vasica* was achieved on MS medium supplemented with varying concentration of IBA after 4 weeks of culture. Media having a low concentration of salts have proven satisfactory for rooting of shoots micropropagation. Roots are mostly induced in the presence of a suitable auxin in the medium. Auxin (IBA) induced root formation which was accompanied by shoot elongation. But it was reported that NAA along with BAP promoted both root and shoot regeneration and finally complete plantlets (Kumar et al., 2003).
II. Quantitative analysis of alkaloids and steroids

Plants are known for the production of a large array of natural products, also referred to as secondary metabolites. They are economically important to man due to their multiple applications, such as pharmaceuticals, flavors, fragrances, insecticides, dyes, food additives, toxins, etc.

The low yield and high market price of the pharmaceutically important alkaloids have created interest in improved alternative routes for their production such as using cell and tissue culture. The callus developed on Murashige and Skoog (MS) media supplemented with different concentrations of auxins and cytokinins was found to have variable alkaloid contents (Verma et al. 2012).

The first attempt for the industrial production of secondary metabolites in vitro was made during 1950 to 1960 by Pfizer Company and the first patent was obtained in 1956 by Routien and Nickell. Several kinds of bioreactors have been designed for large scale cultivation of plant cells. In several cases cell cultures have been shown producing certain metabolites in quantities equal to (Kaul and Staba, 1967) or many fold greater than the parent plant (Zenk, 1978). There were many examples explained the relation between differentiation and secondary metabolites accumulation. Hiraoka and Tabata (1974)
showed that alkaloid concentration in mature plant and callus tissue of *Datura innoxia* was 0.10% and 0.01% of dry weight, respectively (10:1). Also, (Oliveira *et al.* 2001) showed that *Aspidosperma ramiflorum Muell* the ratio of alkaloid concentration between mature plants and morphologically undifferentiated cells of callus was 4:1 (brown friable callus) and 6:1 (yellowish coloured friable callus) increases in total alkaloids concentration.

Regarding secondary metabolites, Solanaceous plants are principally recognized as producers of tropane alkaloids – alkaloids that have also been isolated from *W. somnifera* – in fact, long before the isolation of withanolides from this plant (Khanna *et al.*, 1961). These alkaloids include N-methylpyrrolinium- derived nicotine alkaloids, tropine-derived true tropane alkaloids, and pseudotropine-derived nortropane alkaloids, also called calystegines (De Luca and St Pierre 2000, Griffin and Lin 2000; Drager, 2006).

Because, some of the species of the genus *Withania* exhibited pathological-physiological and biological-activities, their chemistry have been extensively studied, incidentally, very less attention has been paid to study the alkaloid pattern in *Withania* species, in particular, but very few reports have been published on the production of alkaloid in *Withania* species (Dhalla *et al.*, 1961; Schroter and Neumann, 1966; Parr *et al.*, 1990; Pati *et al.*, 2008; Soni *et al.*, 2010; Parvatham, 2011; Bhatt *et
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al., 2011; Sehgal et al., 2012. The presence of high alkaloids levels in *W. somnifera* is responsible for the pharmacological properties and clinical symptoms.

As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow growing plants or difficult to synthesize with chemical methods.

**Effects of different concentrations of auxins and cytokinins singly on callus proliferation and total alkaloid content.**

2,4-Dichlorophenoxyacetic acid (2, 4-D) showed stimulatory effects on callus proliferation and total alkaloid content. Maximum callusing response (76%) was noted at 4.0 mg/l and the minimum at 0.25 mg/l. Of six concentrations of 2, 4-D used in MS media, 2.00 mg/l was better for callus proliferation while 6 mg/l for increase in total leaf alkaloid content. It was noted that the higher concentration of 2, 4-D in media had an inhibitory effect on callus proliferation but stimulated a gradual increase in total alkaloid content. Fresh weight and total alkaloid content in leaf callus at different concentrations of 2, 4-D were significantly higher than for 0.25 mg/l 2, 4-D except for 0.50 mg/l and 1.00 mg/l.

MS media fortified with different concentrations of IBA showed stimulatory effects on callus growth and total alkaloid content. Maximum
callusing response (80%) was noted at 1.5.0 mg/l of IBA and the minimum at 0.25 mg/l. Of six concentrations of IBA used in MS media, a concentration of 1.5-2.00 mg/l was found to be better for increase in total alkaloid content.

**Effects of different concentration and combination of growth hormones on total alkaloids and steroids content**

MS media with 2,4-D and BAP(1.0mg/l+1.5mg/l), 2,4-D were found to be the most promising media in relation to callus biomass increase and content. This increase was also found to be statistically significant. In callus cultures, presence of alkaloids indicates that capacity of synthesis of specific compounds is usually retained during culture (Hiraoka and Tabata, 1974).

**Effects of strength of MS media on leaf callus proliferation and total alkaloid content**

Decrease in strength of MS media had no effects on callus biomass though it enhanced total alkaloid content. Of ten combinations that were used, half strength MS medium with 2, 4-D and BA (0.5 mg/l +1.0 mg/l) was found to be the best for production of adequate callus biomass along with high alkaloid content.

Tissue culture has been suggested as a feasible technology for the production of many plant secondary metabolites. For example,
ginsenoside from *Panax gingseng*; rosmarinic acid from *Coleus blumei*, shikonin from *Lithoserum erythrohizon*, diosgenin from *Dioscorea*, ubiquinone-10 from *Nicotiana tabacum*, berberin from *Coptis japonica* and podophyllotoxin from *Juniperus chinensis* accumulated at much higher levels in cultured cells than in intact plants (Misawa *et al.* 1985; Smith *et al.* 2002; Premjet *et al.* 2002). According to Kalidass *et al.* (2010) the production of secondary metabolites in callus cultures is controlled by environmental factors, viz medium component, pH and temperature. To obtain high leaf callus biomass and alkaloids in higher concentration, *in vitro* experiments were carried out varying the composition of the media.

The varying responses in *in vitro* culture of *Withania somnifera* were noted at different phytohormonal concentrations and combinations from leaf callus. The auxins were found to be the best for callus proliferations and growth. Among auxins, 2, 4-D was better for increase in callus biomass and total alkaloid content. 2, 4-D was also reported as the most effective auxin in various medicinal plants (Junaid *et al.* 2008; Misawa 1994; Asaka *et al.* 1993), while combinations of auxins with cytokinins were found to be better for leaf callus growth and enhancement in alkaloid content. These results are in accordance with the view of Zenk *et al.* (1977) and Brown (1990) that plant growth regulators have
remarkable effects on growth and differentiation and thus metabolism of cultured cells.

The highest enhancement in total alkaloid production resulted from 0.50 mg/l of 2, 4-D and 1.0 mg/l of BA, compared with other combinations. Combinations of 2, 4-D and BA were used by Olivia et al. (2001) for enhancement of ramiflorin in callus of *Aspidosperma ramiflorum*, Taha et al. (2009) for corydalin in callus of *Corydylis terminalis*, Khan et al. (2008) for alkaloid in callus of *Corydylis ophiocarpa*, Yamada & Hashimoto (1982) for tropane alkaloids in callus of *Hyocyamus niger*, Morimoto et al. (1994) for rosmarinic acid in callus of *Salvia miltorizhhiza*, and Mirjalili et al. (2009) for steroidal lactone in callus of *Withania somnifera*.

Our findings indicate that callus growth was found to be more efficient on a MS medium with full strength, rather than half strength which is consistent with the findings of Kadkade (1981, 1982). The results agreed with those of Drewes & Staden (1995) for solasodine production in *Solanum mauritianum* and of Rosli et al. (2009) for 9-methoxycanthin-6-one production in *Eurycoma longifolia* callus cultures. The nutrient concentration of a particular basal medium was also previously reported to be the greatest contribution towards the variation of solasodine production in callus cultures of *Solanum aviculare* (Kittipongpatana et al. 1998).
According to Rhodes et al. (1990) and Drewes and Staden (1995), the capability of different basal media formulation in supporting plant cell growth and the synthesis of plant secondary metabolites were linked to the ionic balance in the medium. Drewes and Staden (1995) and Lipavaska and Vreugdenhil (1996) stated that it is important to find a suitable nutrient concentration of basal medium because lower concentration of nutrient components is not enough to support cell growth and higher concentrations of nutrients may become toxic and cause an osmotic stress for plant cell cultures.

Besides, plant growth regulators and the strength of MS media, various carbon sources and their concentrations were found to have a significant influence on leaf callus growth and total alkaloid content. In general higher concentrations of carbon resources caused an enhancement in callus biomass as well as total alkaloid content. Our study showed that the maximum callus biomass production occurred with 6% glucose while maximum alkaloid production occurred at a concentration of 6% sucrose. This is in accordance with the finding of Woo et al. (1998) that higher concentrations of monosaccharide caused the maximum production of biomass. Studies on callus of *Hyoscyamus niger* showed that increasing sucrose concentration caused an increase in biomass as well as stimulation of production of scopolamine alkaloid (Hilton and Rhodes 1994). In contrast, Schripsema and Verpoorte (1992) showed that biomass production of *Datura stramonium* root culture in sucrose-
enriched growth medium was higher than in a monosaccharide medium, while alkaloid production was highest in a monosaccharide medium. Although 6% sucrose decreased overall callus biomass slightly, the markedly increased production of alkaloid in comparison with other carbon resources makes it the most effective concentration for large scale production of *Catharanthus* alkaloids. This is in line with findings of Scragg & Ashton (1990) in *Catharanthus roseus* callus culture. Shaoxiong *et al.* (1996) reported that higher concentrations of sucrose cause enhancement in steroidal alkaloid production in hairy roots culture of *Solanum aviculare*.

The present study showed that half strength MS basal medium supplemented with 2, 4-D and BA (0.5mg/l+1.0 mg/l) with 6% sucrose was the best for biomass production of leaf callus and enhancement of alkaloid accumulation in *Withania somnifera*. The results indicate that the combination of different basal media, carbon sources and phytohormones could be a useful tool in developing rational strategies to enhance the production of various bioactive molecules in vitro.

Plant cell culture can be established from an impressive array of plant species, including most of those that produce secondary products of commercial interest. Production of a vital callus for certain biological activity has always been demanded by the researchers in this area of knowledge. Many factors effect callus growth and development, the major ones are of genetic background and physiological status, the source, tissue, chemical composition and physical state of the culture.
medium and culture conditions. A common problem is that cultured cells produce only low levels of desired chemicals or do not produce the chemicals at all. Causes of this problem can be gene expression; pathway regulation or precursor availability levels. It was reported that the production level may sometimes be increased by adjusting the culture medium composition, including the salt, carbon source, growth regulators and vitamins or altering the culture condition. It is suggested that the rate of callus growth and its metabolites are inversely related to its chlorophyll content besides nutrients and plant growth regulators that are usually supplied in cell culture.

Nevertheless, *Withania* active components have promising activities, and biotechnological production could offer an alternative to conventional cultivation. Several laboratories have recently developed plant cell and hairy root cultures for the production of the most important bioactive components of *Withania* extracts, withaferin A and withanolide A. Although withanolide production by in vitro cultures is still far from the levels required for economical exploitation, these studies are useful tools to obtain greater understanding of the withanolide metabolic pathway, allowing the application of plant metabolic engineering techniques to improve the biotechnological production of *Withania* bioactive compounds.
III. Analysis of the variants by RAPD techniques

During tissue culture phytochemical variants arise. Foliage and floral variations are also common. RAPD has been used widely and is proved an efficient tool for assessing genetic variability in the tissue culture process (Adhikari et al., 2004; Qin et al., 2006).

RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Sheidai et al., 2010). There are many advantages of this technique such as: a large number of samples can be quickly and economically analyzed using only micro-quantities of material; the DNA amplicon are independent from the ontogenetic expression; and many genomic regions can be sampled with a potentially unlimited number of markers (Damasco et al. 1996; Maria and Garcia, 2000). RAPD markers have been applied to many plant species to evaluate the clonal fidelity and genetic stability of the micropropagated plants. Sahijram et al. (2003) concluded that somaclonal variation may be detected by visual screening or by using molecular marker such as RAPD and AFLP and by cytological studies.

In present investigation different variants have been characterized with the help of RAPD markers. In OPA06 there was 23%
polymorphism, OPA07- 11%, OPA11- 33%, OPA14- 28%, OPA17-37%, OPA19- 50%, OPBO6- 15% and OPC05 shows 20% polymorphism. OPA05, OPA10, OPB01 and OPC12 did not show any polymorphism. The number of polymorphic bands was variable. Variants that generated in in vitro raised plantlets to be analysed in future generations for useful traits like higher alkaloidal and steroidal component.

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