In another experiment, the effects of various pH levels on reserpine production was tested. In this case only roots were used. For this pH was adjusted from 3.0–8.5 using HCl, $Ca(OH)_2$, KOH and NaOH. The results obtained are shown in Table 41.

Low pH level when adjusted with HCl (3-5) had no effect on reserpine content of root and it was found to be 0.2% in all cases. The highest pH from 5.8–8.5 were adjusted using $Ca(OH)_2$, KOH and NaOH. The reserpine content was always higher in root when pH was adjusted with $Ca(OH)_2$. The highest percentage of reserpine in roots was recorded at pH 7.0 level adjusted with $Ca(OH)_2$ and it was 0.22%. For KOH and NaOH reserpine percentages estimated were found to be the same in all the pH levels except in 8.0 and 8.5 pH where KOH gave slightly higher reserpine compared to NaOH.

DISCUSSION

Mass propagation of plant species *in vitro* is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some medicinal plants. Tissue culture in propagation and its importance in conservation of genetic resources and clonal improvement has been described by different authors (Barz *et al.*, 1977; Datta and Datta, 1985; Kukreja *et al.*, 1989).

In vitro methods of plant propagation include shoot culture with proliferation of axiliary or adventitious shoots and callus culture with organogenesis or embryogenesis. Though all plant cells are theoretically totipotent, attempts with any

tissues to get whole plants lead to failure due to lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions. which are essential for proper growth of cells, tissues and organs (Johri, 1982).

In any preliminary study on the tissue culture of plants, it is customary to use juvenile tissues rather than those from mature plants. This is because most plant populations are highly heterogeneous, the tissues are difficult to grow, and if they grow, they also show high variability in culture responses. Mature plants are also less responsive when compared with juvenile tissues (Sommer and Caldras, 1981). Moreover, juvenile plant parts regenerate adventitious shoots relatively easily. For example, with gymnosperms, adventitious shoot formation is often only possible with embryos, seedlings or parts of seedlings and not mature plants.

In the present study with *R. serpentina*, materials from young branches of field grown plants were used for primary establishment of cultures. For shoot proliferation, shoot tips and nodal explants from 8-9 months old field grown plants were used as explant sources. Explants from actively growing shoots at the beginning of the growing season generally give the best results for enhanced axillary branching (Yang, 1977). Hence, explants were taken from young, newly formed shoots of the plants. These explants were placed on media with different concentrations of cytokinins, with or without auxins. Cytokinins have been shown by many workers to release lateral buds from apical dominance (Rubinstein and Nagao, 1976).

Stimulation of axillary shoots from single nodes proved to be most prolific source of shoots for many varieties of citrus (Barlass and Skene, 1982). In this study, nodal explants as well as shoot tips were cultured on MS basal medium supplemented with different concentrations of BA (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg /l).

Although exogenous auxins do not promote axillary shoot proliferation, culture growth may be improved by their presence. One of the advantages of adding auxins at low concentration to the culture media is to nullify the effect of the higher concentration of cytokinin on axillary shoot elongation (Hu and Wang, 1983).

In the present investigation, the callus formation at the basal portion of the nodal explants and shoot tips inhibited growth of the axillary buds in a high percentage of cultures. It was observed that the frequency of callus formation was greater in cultures of shoot tips than nodal explants. Formation of base callus significantly decreased the frequency of multiple shoot formation, number of shoots per explant and also shoot length. On average, more than 4 shoots from one explant were produced in media having 2.0 mg/l BA + 0.2 mg/ l NAA. This proliferation rate was maintained up to 10^{th} subculture. Some of the shoots grew rapidly and others grew slowly. However, the regenerated shoots reached to a height of about 5 cm if the cultures were maintained for 45 days. It was observed that increasing the BA concentration increased the percentage of explant produced shoots and the optimum concentration was 2.0 mg / l irrespective of concentrations of NAA used (Table 22). When more than 2.0 mg / l BA was used, the percentage of explant responded to multiple shooting decreased and at a certain stage the response was completely ceased (Table 22). This implies that high concentration of BA was deleterious to the explants. Dunstan *et al.* (1985) reported that although increasing exogenous BA induced axillary bud break, high concentration of BA could be detrimental to shoot quality and smaller and unusable shoots were produced. Yadav *et al.* (1990) and Zaman *et al.* (1992) in mulberry and Banu *et al.* (1997) in *Adhatoda vasica* also reported that 2.0-mg / l BA was more effective for shoot multiplication and elongation.

Using GA_3 with BA and Kn did not increase the number of shoots per explant like BA with NAA and Kn with NAA (Tables 23 & 25). Optimal concentration of GA_3 was 0.1 mg / l with 2.0 mg / l BA and 0.5 mg / l with Kn. However, in GA_3 supplemented medium, shoot enlarged and grew healthy with several well-developed leaves within 3–4 weeks of culture. This result contradicts with Smith and Kefferd (1964) who reported that GA_3 appeared to be responsible only for the breaking of dormancy. But in *Atriplex*, GA_3 was found to be effective not only in stimulating shoot elongation but also in enhancing shoot number.

The most effective factor for shoot multiplication from either lateral or apical meristem is auxin-cytokinin combination (Wochok and Sluis, 1980). In many cases, GA_3 is also added to the media. Boxus *et al.* (1977), however, reported that addition of GA_3 in culture medium inhibited shoot proliferation in strawberry. Bhojwani and Razdan (1983) suggested that the application of GA_3 promotes budding. The endogenous level of the hormone must be suboptimal.

The optimal BA concentration for shoot proliferation was studied carefully. In the initial culture media when BA was used singly; optimal concentration was found to be 3.0 mg / l, whereas in the subsequent subcultures comparatively low concentration of BA was beneficial. In pear plants, 2.2 mg / l proved to be the most effective concentration of BA for shoot tip culture (Lane, 1979). However, stem of *Musa* could be induced to form multiple shoots most effectively on media with high concentration (5.0 mg / l) of BA (Cronauer and Krikorian, 1984a,b).

Although some explants on a particular medium showed good response, others on the same medium did not respond in the same way. Three factors may be involved: physiological status of the explants, the size of the explants and quality of the plant from which the explants were obtained (Murashige, 1974; Hu and Wang,

1983). Although these parameters were kept constant while choosing the explants, it was not possible to ensure that all the explants were exactly similar. Collection of explants at different periods of the year could also bring about some variations in response.

Many of the problems of inducing callus from plant tissue may be over come by using parts of freshly germinated plantlets ensuring tissue fragments composed of cells with high potential (Yeoman and Forche, 1980). Only younger parts of the growing shoots were selected as explant source to induce callus and axillary shoots in this investigation.

Though all the plant cells are theoretically totipotent, attempts with many tissues to get whole plants lead to failure due to lack of proper techniques and insufficient knowledge about nutrient media and other physical and chemical conditions, which are essential for proper growth of cells, tissues and organs (Johri, 1982). Callus is an actively dividing non-organized tissues of undifferentiated and differentiated cells often developing from injury or in tissue culture (Pierik, 1987). An exogenous supply of growth hormones is often recommended to initiate callus from different explants. Proper combinations of auxin-cytokinin to the medium are essential for callus induction but their requirements depend strongly on the genotype and endogenous hormone content of explants (Pierik, 1987). Rao and Lee (1986) reported that intermediate levels of auxin and cytokinin usually promote callusing. But many other factors like, genotype, composition of the nutrient media, physical growth factors, such as light, temperature, moisture etc., are also important for callus initiation (Pierik, 1987).

Although major emphasis has been given on Angiosperm tissues, callus initiation has been observed in gymnosperms, ferns, mosses, and liverworts (Yeoman,

1970; Yeoman and Macleod, 1977). Recently, many reports are available on callus initiation from herbaceous and medicinal and vegetable crop plant species (Kamat and Rao, 1978; Fassuliotis *et al.*, 1981: Bhat and Fassuliotis, 1981; Matsuoka and Hinata, 1983; Misra *et al.*, 1983; Jain *et al.*, 1985; Nishio *et al.*, 1987; Saxena *et al.*, 1987; Sihachakr and Ducreux, 1987; Akram and Ilahi, 1985, 1986; Ilahi and Akram, 1987; Ilahi *et al.*, 1988; Sarker *et al.*, 1996; Roy *et al.*, 1994, 1995; Banu *et al.*, 1997).

The present study on callus induction was conducted with internodes and leaves of *R. serpentina* to observe their callus induction capacity. MS medium supplemented with different auxin-cytokinin combinations was used to study the callusing response. Two types of plant materials were used; field grown plants and *in vitro* grown plants. 2,4-D, NAA, IBA and IAA were used as auxins and BA and Kn were used as cytokinins in different concentrations. It was found that callus proliferation strictly depended on exogenous hormone supplementation. In absence of exagenous hormone, explants failed to induce callus and became necrotic and died within a few days.

Among the four auxins used, 2, 4-D was found to be the best in respect of callusing response. On the other hand, BA was superior to Kn for callus growth when supplemented with 2, 4-D (Table 27). Normally, lower concentrations of cytokinin in combination with higher concentration of an auxin was favourable for callus induction. There are well documents on callus proliferation when auxins were used alone or in combination with cytokinin (Rao *et al.*, 1981; Lee *et al.*, 1982; Hammerschlag *et al.*, 1985; Akram and Ilahi, 1985; 1986; Ripley and Preece, 1986; Jain *et al.*, 1988; Espinasse *et al.*, 1989; Niedz *et al.*, 1989; Verhagen and Wann, 1989; Roy and De, 1990; Banu *et al.*, 1997).

In the present investigation, it was observed that callus initiation started from the cut surfaces of the explants within 4 to 5 days of culture and the whole explants turned into callus masses within 12 to 15 days. Calli derived from the both explants (internode and leaves) were soft, but calli derived from internodes were white and those from leaves were green. When the auxins 2, 4-D, NAA, IBA and IAA were used separately, maximum 83.33% of the cultures responded to callusing in 3.0 mg / l 2,4-D, whereas 41.66% cultures responded to callus in 3.0 mg / l NAA, 4.0 mg / l IBA as well as 4.0 mg / l IAA. The highest fresh weight of callus (3. 64 g / culture) was obtained in 3.0 mg / l 2, 4-D (Table 27).When BA and Kn were added separately to 2, 4-D supplemented media, callus induction started within 2 to 3 days and the explant turned into callus masses within 8 to 10 days. One hundred percent of callus induction was observed from internode explants in 2.0 mg / l 2, 4-D+0.5 mg / l BA (Table 28). The callus weight was further increased to about 4. 54 g per culture when 0.1 mg / l CH (casein hydrolysate) was added to the medium (Table 30).

On the other hand, when leaf segments were cultured with different auxins, 66.66% of cultures responded to callus in 3.0 mg / l 2, 4-D and maximum fresh and dry weights of 2.69 and 0.12 g per culture, respectively was achieved. The percentage of culture and fresh and dry weights were further increased in 2.0 mg/ l 2, 4-D+0.5 mg/ l BA + 0.5 mg/ l CH (Table 30). Therefore, the combination of 2,4-D and BA was the most effective formulation for both explants used in the induction of callus and growth of callus, whereas IBA alone or either with BA or Kn was the least effective to induce callus formation. Oka and Ohyama (1976) got better results in medium with 2,4-D than IAA or NAA in mulberry which is in agreement with the present findings. Illahi and Akram (1987) also reported that 2,4-D used alone was considerably effective for callus formation in leaf segments of *R. serpentina* at various concentrations ranging from 0.1 to 1 mg/ l than NAA. These results are in

agreement with Staba (1969) who found 2,4-D alone as an effective auxin mostly at a concentration of 1 mg/ l.

Many workers observed 2,4-D as the best auxin for callus induction as common in monocot and even in dicot (Evans *et al.*, 1981; Lu *et al.*, 1982; Ho and Vaxil, 1983; Litz, 1984; Jaisal and Narayan, 1985; Chee, 1990). Kamat and Rao (1978) reported that 2,4-D which was most potent in stimulating the callus growth of explants proved in effective when used alone. This statement is also in agreement with the present findings. However, the present results differed with the work of steward *et al.*, (1958) who observed that elimination of 2,4-D from media induced callus proliferation in carrot cell cultures. This result also contradicts with the report of Medora *et al.*, (1979) who demonstrated inhibitory effect of BA in combination with 2, 4-D on induction and growth of callus in papaya.

Shoots obtained *in vitro* have to be rooted to obtain complete plants. In the present investigation, it was observed that field grown explants failed to induce root formation when placed in a culture medium. Hence, shoots obtained from *in vitro* grown plants were subcultured in MS basal medium supplemented with different concentrations of auxins, NAA, IBA and IAA alone and in combination. Shoots obtained from first and second subcultures failed to induce root formation. Rooting of *R. serpentina* started only when explants were taken from third subcultures. Most frequently, root formation is inhibited by the cytokinin used to induce shoot multiplication, so that the shoots do not produce roots *in vitro* until they were cultured on a medium containing auxins (George and Sherrington, 1984). Generally, rooting was induced within 10 to 15 days of transfer to root inducing medium for both explants (internodal segment having one axillary bud and shoot tips).

In the present experiment, NAA was superior to other auxins when used singly or combinedly with the other auxins for rooting. Among the different concentrations tested, 1.0 mg/l NAA was found to be the best concentration for root induction and growth of roots. The combination of IBA and IAA (3.0 mg/l IBA + 0.5 mg/l IAA) was more effective for rooting than any concentration of single IBA or IAA. One hundred percent of root cultures responded to rooting in 1.0 mg/l NAA when the explant source was shoot tips.

The percentage of cultures responded to rooting was always higher in shoot tip explants than nodal explants having one axillary bud. This result is similar to the result of Mathur *et al.* (1987) who reported that 1.5 mg/ l NAA was the best for root induction among all other auxins for *R. serpentina*. The number of roots per explant, the length of roots and fresh and dry weights of roots were higher in 1.0 mg/ l NAA. Roy *et al.* (1985) also reported that 1.0 mg/ l IBA + 1.0 mg/ l IAA was the best combination of auxins for proper rooting in *R. serpentina* and 100% shoots rooted within four weeks of culture. Roots began to emerge from tenth day of culture and within a period of 23–28 days 100% shoots rooted. Banu *et al.* (1997) observed that the average number of roots of *Adhatoda vasica* was better in NAA than those of IBA and IAA and even in NAA + IAA formulation. In 2.0 mg/ l NAA, the highest average number of roots was found to be 6.00.

The results of the present investigation clearly show that both the shoot tips and nodal explants of *R. serpentina* plant are capable of producing multiple shoots *in vitro* and subsequentljy root to form complete plantlets. After acclimatization, the plantlets were planted in the field where 80% of plants survived.

When all the steps of micropropagation of *R. serpentina* had been optimized, reserpine content in the different types of cultures was studied. During the study of

alkaloid content in the different parts (roots, leaves, stems) of *in vitro* grown plant, alkaloid content of field grown plant was also studied as a control. In order to know and compare the content of alkaloid in shoots and leaves of *in vitro* grown plants, the stem and leaves of field grown plants were also studied.

Roots of *R. serpentina* contain 0.15–0.20% reserpine–rescinnamine group of alkaloids (Anonymous, 1969). In the present study, root has been found to contain 0.192% reserpine in 8 – 9 months old field grown plants. The *in vitro* grown plant materials used for assay of reserpine content were collected from the cultures of the 4th subculture. It has been previously reported by Marta *et al.* (1992) that alkaloid production increased after 4th subculture. Alkaloids are accumulated in the cell when the growth of cells stopped (Marta *et al.*, 1992). Considering this idea, different plant parts were collected after 8 weeks of 4th subculture or subsequent subculture. After 8 weeks of any subculture, growth of roots was found to be decreased or stopped. Alkaloid content varied greatly among the different types and concentrations of phytohormones. Significantly greater amount (0.22%) of reserpine was obtained from the roots grown in the medium when NAA was supplemented than from IAA or IBA. Further increase in reserpine content was achieved while pH of the medium was raised to 6.0–7.0 by adjusting with $Ca(OH)_2$. The roots in this medium were hairy. Hairy roots were found to contain more alkaloid than smooth ones (Hamill *et al.*, 1986; Mano *et al.*, 1989; Verpoorte *et al.*, 1991 and Pyne *et al.*, 1987). Superiority of NAA over other auxins for the metabolites has also been reported by other investigators like Tabata *et al.* (1971) in tobacco, Tabata *et al.* (1974) in *Lithospernum erythrorhizon*, Zenk *et al.* (1975) in *Morinda citrofolia*. The alkaloid content of roots of media supplemented with other two auxins (IBA and NAA) was significantly low.

There was little amount (0.05%) of reserpine obtained from both *in vitro* and field grown shoots. Roja *et al.* (1980) also claimed that little amount of reserpine–like alkaloid, ajmalidine, was present in shoot culture. In the present study, the alkaloid has been obtained as reserpine. No variation was observed in the content of reserpine obtained from shoots cultured in different hormonal combinations.

In the present investigation, internode and leaf derived calli induced in medium supplemented with 2, 4-D, BA and CH were used. The amount of reserpine obtained from the calli ranged from 0.08–0.09%. Similar results were obtained by Heijden *et al.* (1989) in *Tabernaemontana elegans*. Illahi *et al.* (1988) analysed root, stem and leaf calli for *Rauwolfia* alkaloids. Ajmaline was the major alkaloid produced by the cultures. Alkaloids in such plants were higher in leaf and stem cultures than the parent plant.

The alkaloids produced by the *in vitro* grown plants may be secreted into the culture media where they were grown. Having this assumption, all the nutrient media from which plant parts were used for alkaloid extraction were also analyzed for the presence of reserpine. In all the cases reserpine was found to present in the culture media irrespective of type and concentration of phytohormones, but the amount was very low (0.03-0.04%). Yamamoto *et al.* (1987) also demonstrated the presence of alkaloid, berberine in tissue culture media of *Thalictrum minus*.

Now the question arises why does NAA supplemented media contain high amount of reserpine? When the pH of the medium was raised to 6-7 by adding Ca $(OH)_2$ to the medium, it further increased reserpine content. Dicosmo and Towers (1984) made an extensive review regarding the effect of different factors, like temperature, pH, nutrition (vitamins, minerals, etc) or phytohormones on secondary metabolite production in cultured plant cells.

In the present study, it was not possible to estimate the production of reserpine considering all the factors individually. However, it could be concluded that the auxin NAA had a stimulating effect in the reserpine production. Further, it may be assumed that at neutral pH range, any of the alkaloid biosynthetic enzymes is activated by Ca^{++}, which has wide activity on enzymatic level. Peroxidase enzymes have been reported to play a role in the biosynthesis of indole alkaloids (Smith *et al.*, 1988; Goodbody *et al.*, 1988a, b, c; Endo *et al.*, 1987; 1988). Ca^{++} has been found essential for maintaining the protein structure in the heme environment of the horseradish peroxidase (Ogawa *et al.*, 1979) and it has also been reported to have great influence on the peroxidase (Sticher *et al.*, 1981; Kevers *et al.*, 1982).

In tissue cultures, the known alkaloids are reported as altogether absent or when present are in traces. This behaviour is well reasoned and documented by various researchers (Luckner *et al.*, 1977; Hirotani and Furuya, 1977; Bauch and Leister, 1978). Although secondary metabolites may be produced in large quantities by cultures but the type of compound produced is often unexpected or even novel and specific compounds of the species might not be produced at all. Stockigt *et al.* (1981) were able to screen 10 previously not reported alkaloids from cell cultures of *R. serpentina.* The yield of vomilenine was 51 times higher in cultures than that found in differentiated plants.

This does not necessarily imply that best known plant products or particular secondary metabolites can only be produced by organized cell system but suggests that cells growing *in vitro* under arbitrary conditions do not always produce a characteristic secondary metabolite, particularly when reverted to a more basic mode of existence of replicative growth with conditions approaching favourable for growth of microorganisms.

However, new techniques are available to modify and improve the biosynthetic potential of cultured plant cells. In some plant species, cultures contain higher contents of secondary metabolites than intact plants e.g. pseudo-phedrine from *Ephedra foliata* is much higher (2.25%) in suspension than that in stem (Khanna *et al.*, 1976). Production of diosgenin in *Trigonella foenum-graecum* cultures was found at 1.82% by Khanna and Jain (1973) as compared to seeds containing 1.6%. By the use of 50 mg/100 ml cholesterol in culture media they were able to increase diosgenin content to 3.54%. Solasodine was increased up to a maximum of 680 µg/g dry weight with 70 mg/100 ml cholesterol in cultures of *Solanum xanthocarpum* (Khanna and Manot, 1976). Feeding of ascorbic acid to cultures of *Tagetes erecta* increased pyrethrin contents (Khanna and Khanna, 1976). Similarly, Khanna *et al.* (1978) were able to increase the *in vitro* production of major opium alkaloids with 12.5 mg/100 ml tyrosine. In *R. serpentina*, feeding experiments are not known except nutritional additives as coconut milk (Mitra and Kaul, 1964) or yeast extract (Ohta and Yatazawa, 1979) and casein hydrolysate (Ilahi *et al.*, 1988). Nevertheless, the cultures produced 96. 23^% more ajmaline in dark when compared to light grown cultures (Ilahi and Akram, 1987). Therefore, it is assumed that new alkaloids of *R. serpentina* cultures could be produced. This presumption is well supported by the work of Stockight *et al.* (1981) and Schubel and Stockight (1984).

Recently a rather new technique using enzyme systems for regulating biosynthetic potentials in *Rauwolfia* cultures is given by Stockight (1984) and Pfitzner *et al.* (1984).

With these techniques and improvements, plant tissue and cell culture systems could play an important role in the manufacture of biological compounds of therapeutic value.