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IN VITRO REGENERATION AND FIELD TRANSFER OF *RAUWOLFIA* PLANTS

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Root callus of *Rauwolfia serpentina* was induced to differentiate buds with 0.8 mg/1 NAA and 2 mg/1 BAP. Buds were rooted with 24 hr treatment of IAA + IBA (both 3 mg/1). Rooted buds differentiated into autotrophic plantlets. The plants on transfer to soil thrived well and matured successfully to produce flowers and fruits. Karyotype analysis of flower buds showed pollen mother cells as having 22 chromosomes (2n), as in cultivated *Rauwolfia* plants.

Key words: Root tissue, Plant regeneration, Field growth.

Introduction

Rauwolfia serpentina Benth., is an important medicinal plant. Its root is used as a raw material for isolation of alkaloids reserpine, ajmaline, serpentine and ajmalicine etc. The drug is collected from wild plants growing in tropical Asia. The cultivation of *Rauwolfia* plant is very difficult for various reasons, one being formation of a large proportion of non-viable seeds [1]. Consequently, propagation of *R. serpentina* by tissue culture has been suggested by various workers [2-4].

Field plantation of *In vitro* regenerated plants of most plant species is often a problem [5-7]. The object of present study was, therefore, to observe the behaviour on field trials of root regenerated plants of *R. serpentina*. Such work is important for establishing tissue derived plants in open agriculture lands, especially for woody species, which are difficult to culture, but are of great economic and medicinal value.

Materials and Methods

Culture conditions. White's Root Culture (WRC) medium [8] was used in this study. Sucrose was used as carbohydrate source at 1.5%. The pH of media was adjusted at 5.6. Coconut Milk (CM) prepared as reported in an earlier communication [9] was added to the culture media at 100 ml/1. A stock solution of extra nitrogen (EN) was made by dissolving 0.32 gm of calcium nitrate and 10 gm of potassium nitrate in 100 ml of distilled water. Ten ml of the stock was added to 1 liter of medium. Cultures were kept at 28° with 16 hr fluorescent light. Subculturing was carried out regularly at 4 week intervals.

Green house conditions. Soil used in pots was a mixture of sandy loam and decomposed leaf manure in equal quantities. Knops solution prepared after Gautheret [10] was diluted with equal amount of distilled water, to make it half strength. Humidity covers made of 0.2 mm thick, transparent polyethlene sheet were used to maintain humidity around potted plantlets.

Illumination was by diffused day light from glass walls. Temperature for day and night was kept different, as follows : 32 (days)/ 25° (night) (Aug.- Sept.); 29 (day)/17° (night) (Oct.); 22 (day)/9° (night) (Nov.), except for Dec.-Feb., which was kept constant at 28° for day/night.

Field growth conditions. The atmospheric temperature in the field during these studies was an average maximum of 33.21, 36.39, 30.54 and 18.09° respectively, for spring (Apr.-May); summer (June-Aug.); autumn (Sept.-Nov.) and winter (Dec.-Feb.) months. The average minimum temperature for these seasons was 15.42, 24.91, 13.78 and 6.85° respectively. The plantlets were protected from direct sun after mid-day in summer and from frost in winter nights, by covering them with temporary shelters made of wooden boards.

The soil of the experimental field was of clay origin. It was made more open by the addition of sand and decomposed leaf mould at the rate of 20 and 12 kg respectively per square meter of soil. Soil pH from surface level upto a depth of 24.5 cm was found to be in the range of 7.87 to 8.06 (average 7.97). Irrigation of the field was done with canal water (pH 7.91) once a week in summer and once a fortnight or as required, during winter months.

Karyotype analysis. Karyotype analysis was carried out according to the methodology reported in an earlier communication [11].

Results and Discussion

Shoot regeneration. Rauwolfia roots were cultured on WRC medium containing 10% CM, 10 mg/1 biotin and either (a) 10 mg/1 BAP + 1 mg/1 IAA or (b) 2 mg/1 BAP + 0.8 mg/1 NAA and 10 ml /1EN. Callus swellings were observed at 5 weeks on medium (a) which later produced good amount of callus in the following 10 days (Fig. 1). Swelling with some initiation of callus was observed on medium (b) in 6 week. The calli in both cultures were compact, hard textured and green in colour.

Both types of calli were subcultured on their respective induction media (a and b). The calli showed copious growth but remained undifferentiated. The callus growth on medium (a) was rather slow. Therefore, in subsequent subcultures it was provided with 10 ml/1 EN, as in the case of callus on

medium (b). Therefore, both calli grew favourably on their respective media.

In the 14th week of growth, root callus on medium (b) exhibited differentiation of buds where as callus on medium (a) remained undifferentiated for the same period. Therefore, the callus growing on medium (a) was also transferred to medium (b) and shoot differentiation was then observed in the following 15 days. Buds were observed as green protuberances, 1-2 per callus inoculum. Differentiated buds were left for 15 days for further development (Fig. 2) and were then excised and cultured onto fresh medium (b), where they were left to harden over in 45 days.

For rooting, buds were placed on filter paper platforms, in liquid basal WRC (BM), after giving a 24 hr treatment with 3 mg/1 IAA + IBA under sterile conditions. The percentage rooting of treated buds on BM was found to be 14.3 after one week. Growth was observed in 100% of the buds and shoot elongation in 43%. The rooted buds showed sufficient shoot growth (Fig. 3) to be transferred to soil over the next 3 weeks.

Green house observations. In green house, with diffused day light dark green colour of plantlet's leaves changed to light green colour in 2-3 days time. The plants grew normally otherwise for the next 4 months and attained an average height of 87 mm. The plants tolerated low night temperatures until, it was lowered to 9° or less. Then plants showed symptoms of winter injury i.e. leaves turning yellow, shedding of older one's and very slow growth in general. Therefore, the green house temperature was raised to stay constant at 28° and plants showed activity of the apical buds and resumed growth after one week. Shoots were seen to lengthen and new large, wide and healthy leaves started apearing over the next 5 weeks.

Natural dormancy found in cultivated counterparts at that time of the year (Dec.-Jan.) seemed not to be exhibited by regenerated plants. The average height of plants increased and was noted as 138 mm in 5 week. Leaves looked healthy and normal; older leaves were darker than the new one's which were of pale green colour (normal character); leaves were normal in shape, long and lanceolate as of cultivated plants. Growth in apical buds was clearly visible. In 14 weeks total growth with constant day/night temperature, the plants attained an average height of 214 mm.

The plants at this stage were subjected to varied day/night temperature of 25/12°. Humidity covers were removed and sun-burn spots were noted on leaves immediately in new growth conditions. However, these disappeared after one week. Growth was observed, however to be rather slow. The plants attained an average height of 220 mm in the next 3 weeks.

Field growth. The plants from the green house were transferred directly to open field conditions, in plots. Transfer

FIg. 1. Callus formation on *Rauwolfia serpentina* root, cultured on WRC medium with 10% CM, 10 mg/1 biotin, 10 mg/1 BAP and 1 mg/1 IAA.

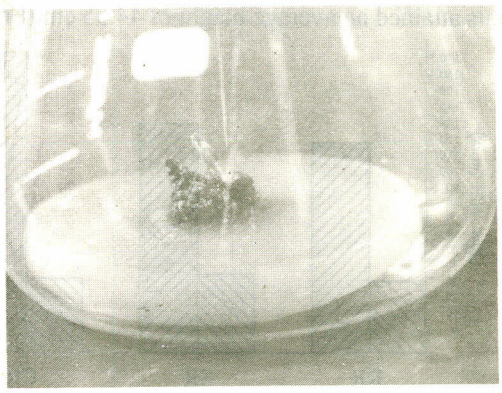

Fig.2. Shoot bud differentiation in *Rauwolfia* root callus on WRC medium containing 10% CM, mg/1 biotin, 2 mg/1 BAP, 0.8 mg/1 NAA and 10 ml/1 EN.

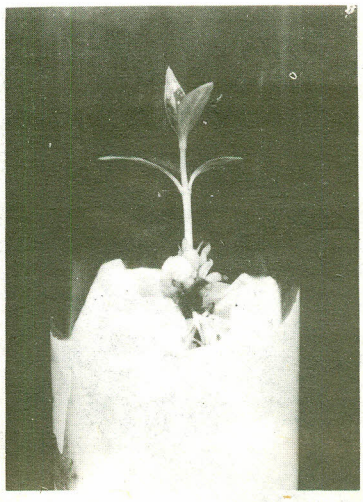

Fig. 3. Root formation in differentiated *Rauwolfia* buds on BM (WRC) medium and subsequent enhanced shoot development.

of plants was carried out in mid-April; the average atmospheric temperature for day/night in the month of April was 32.72/12.33°. Further details on atmospheric day/night average temperature for subsequent months seasons, when plants were growing in field is given under M. and M. Section.

During 4 weeks of growth, the plants seemed to be in good condition, with some growth of leaves and apical buds. The plants attained an average height of 255 mm. The general appearance of the plants was normal, healthy and similar to cultivated *Rauwolfia* plants. In the following 2 weeks, an average of 2 flower buds per plant, appeared at the 13/14 and 16/17 nodes. Similarly, vegetative growth also continued progressively in these two weeks. The following 2 weeks, the plants almost doubling in height to an average of 430 mm. By this time, pollination was also observed to take place in opened flowers. The leaf colour, shape and size was almost comparable to conventially cultivated *Rouwolfia* plants (Fig .4). Growth continued over the following 4 weeks and the plants attained an average height of 44.55 cm (Fig. 5).

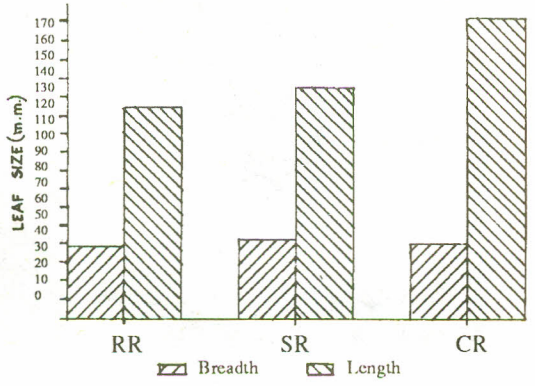

Fig.4. Histogram showing average size of a representative leaf from root regenerated (RR), stem regenerated (SR) and cultivated *Rauwolfia* plants (CR).

Fig.5. *In vitro* regenerated *Rauwolfia* plant growing in field (soil). Notice the production of flowers.

Thereafter, in 2 months, plants attained an average height of 62.55 cm. At this stage, branching was also observed in most of the plants, pollinated flowers produced seeds. The first batch of ripe seeds was collected after 4-5 months of flowering (Fig. 6).

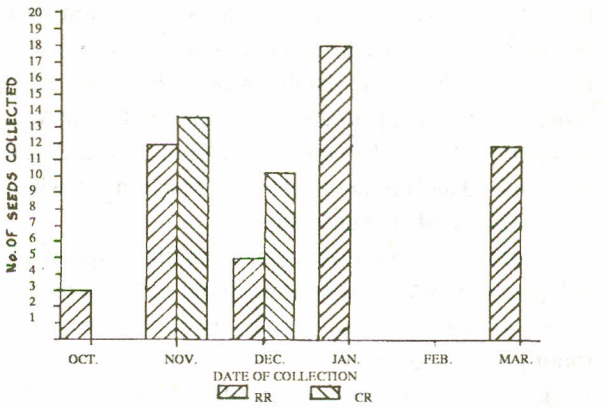

Fig.6. Histogram showing the average number of ripe seeds collected in late season, from root callus regenerated (RR) plants and cultivated *Rauwolfia* (CR) plants.

Karyotype analysis. Pollen Mother Cells (PMC) from regenerated plants show 11 bivalents at metaphase (Fig. 7a). In another cell, 11 univalents are seen moving to each pole at anaphase (Fig. 7b); confirming thereby that the regenerated plants have (2n = 22) chromosomes. In comparative analysis of PMC's from cultivated plants showed 11 bivalents at M-1 (Fig. 7c). The same number of chromosomes was confirmed at anaphase (Fig. 7d), where 11 chromosomes were seen moving to each pole after separation.

From the results obtained in the present study it was found that callus initiation on root explants of *Rauwolfia serpentina* and further proliferation of the callus was possible with the manipulation of nutritional and environmental conditions. Root callus techniques for *Rauwolfia* were also reported in our earlier communications [9,12].

Shoot regeneration in *Rauwolfia* root callus required lowering of the BAP concentration in the medium. A high concentration BAP (10 mg/1 - medium a) favoured an undifferentiated growth of callus, while a comparatively low concentration (2 mg/1-medium b) allowed bud regeneration. In a related work on shoot regeneration in stem callus of *Rauwolfia* [13] an exogenous supply of BAP was not required at all. This may be due to there being another source of cytokinin available to the regenerating callus as e.g. Coconut Milk (CM), in the present study. CM is reported to contain various growth regulators, including cytokinins [14].

Root formation in regenerated buds was not spontaneous e.g. experienced in *Rauwolfia* buds regenerated from stem callus [13]. Instead the buds rooted only when IAA

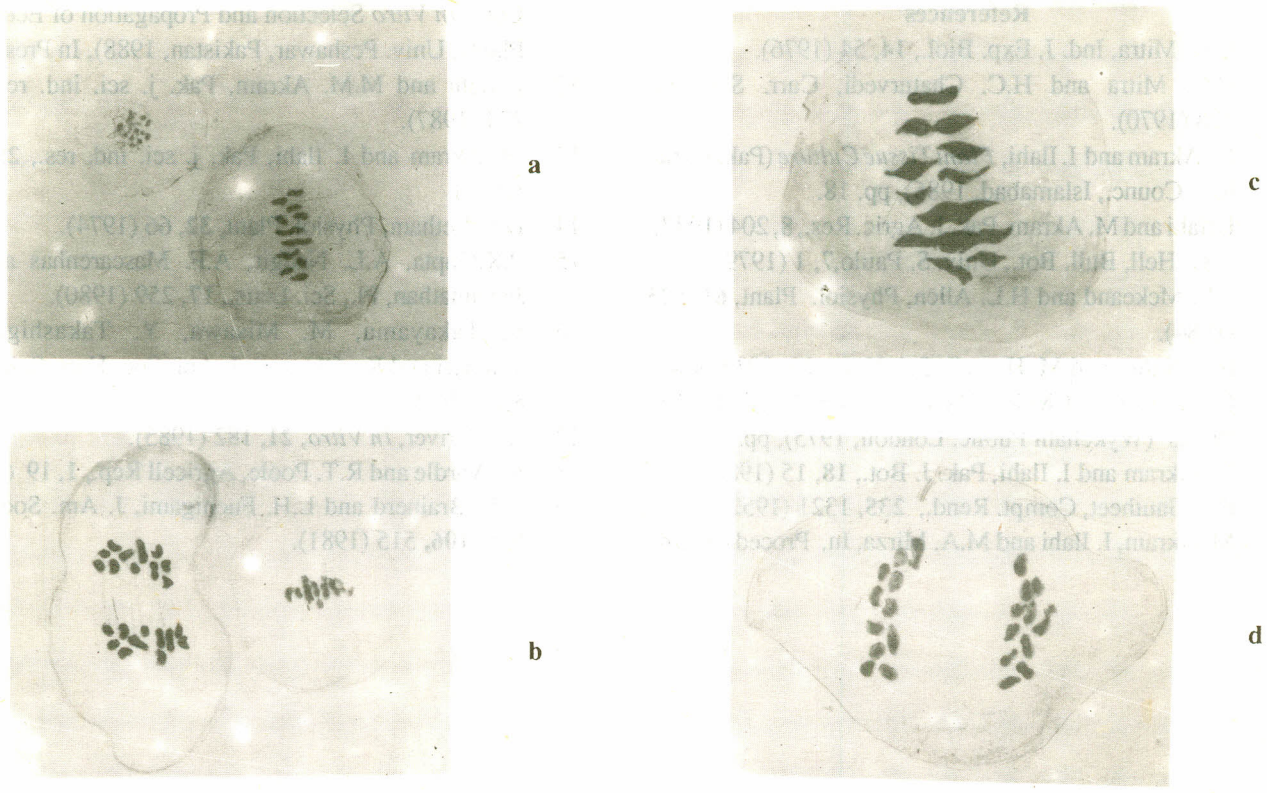

Fig.7. Pollen Mother Cells (PMC's) of regenerated (**a-b**) and cultivated (c-d) *Rauwolfia* plants. (**a,c**) at M-1 stage showing 11 bivalents, (**b,c**) at anaphase showing (2n = 22) chromosomes.

and IBA were given in combination. Similarly combined effect of IAA, IBA with IPA was also reported in regenerated buds of *Tectona grandis* by Gupta *et. al.* [15].

The adaptability of regenerated plants from flourescent lighting to sunlight was gradual. The plants on transfer to the field did not show any ill-effects and were growing normally and very similarly to cultivated *Rauwolfia* plants, except for temporary leaf wilting in direct sunlight. The observations mentioned in this study commonly occur in field planting of various regenerated plants as reported by other workers [16-17]. Wardle and Poole [18] indicated that the high humidity conditions of aseptic culture result in a lesser depth of palisade cells and inhibition of surface wax production. This may be reason for temporary wilting during sunlight in the field. However, this wilting was less pronounced in root tissue regenerated plants of *Rauwolfia* as compared to stem tissue regenerated plants [11]. The *Rauwolfia* plants required low humidity. Similar results have also been reported for other woody species e.g. apple [19]. This helped in the establishment of regenerated plants in relatively low humidity in open fields.

The regenerated *Rauwolfia* plants produced flowers, fruits and seeds. The normal collection month for ripe seeds of *Rauwolfia* plants is July. But as the regenerated plants started flowering late in June, may be due to interference with the natural photoperiod under green house conditions, the ripe seeds were not available in July and subsequently developed in later months, even upto March. As compared to late season seed collection from conventionally cultivated plants, the seeds produced by root tissue regenerated plants were only 45.62%. However, this does not necessarily mean that low seed production is the characteristic of tissue regenerated plants as e.g. *Rauwolfia* plants regenerated from stem callus [11] produced 118.34% more seeds then cultivated *Rauwolfia* plants (data for late season seed collection).

Cytologically there seemed to be no quantitative differences in chromosome number analyzed both from callus regenerated and cultivated *Rauwolfia* plants. The results in Fig. (7a-d), there appeared to show slight variations in chromosomal shape at M-I and anaphase; and chiasmatae frequencies at M-I in both sources. These differences did not induce any changes in the morphology and fertility of the plants derived from callus as proved during the present studies.

Therefore, the results given here strongly advocate that callus regenerated plants of *Rauwolfia serpentina* can be safely planted in field and could be grown to maturity like the cultivated *Rauwolfia* plants.

References

1. G.C. Mitra, Ind. J. Exp. Biol., **14**, 54 (1976).
2. G.C. Mitra and H.C. Chaturvedi, Curr. Sci., **39**, 128 (1970).
3. M. Akram and I. Ilahi, *Plant Tissue Culture* (Pak. Agric. Res. Counc., Islamabad, 1985), pp. 18.
4. I. Ilahi and M. Akram, Pak. J. Agric. Res., **8**, 204 (1987).
5. K.G. Hell, Bull. Bot., Univ. S. Paulo,**7**, 1 (1979).
6. S.E. Mckeand and H.L. Allen, Physiol. Plant, **61**, 523 (1984).
7. E.G. Sutter and M. Hutzell, Sci. Hort., **23**, 303 (1984).
8. E. Thomas and M.R. Davey., *From Single Cell to Plants* (Wykeham Public. London, 1975), pp. 149.
9. M. Akram and I. Ilahi, Pak. J. Bot., **18**, 15 (1986).
10. R.J. Gautheet, Compt. Rend., **235**, 1321 (1952).
11. M. Akram, I. Ilahi and M.A. Mirza, In, Proced. Intern., Conf. *In Vitro* Selection and Propagation of Economic Plants, Univ. Peshawar, Pakistan, 1988), In Press.
12. I. Ilahi and M.M. Akram, Pak. j. sci. ind. res., **30**, 224 (1987).
13. M. Akram and I. Ilahi, Pak. j. sci. ind. res., **28**, 412 (1985).
14. D.S. Letham, Physiol. Plant, **32**, 66 (1974).
15. P.K.Gupta, A.L. Nadgir, A.F. Mascarenhas and V. Jagannathan, Pl., Sci. Letts., **17**, 259 (1980).
16. S. Takayama, M. Misawa, Y. Takashige, H. Tsumeri and K. Ohkawa, J. Am. Soc. Hort. Sci., **107**, 830 (1982).
17. J.A. Driver, *In Vitro*, **21**, 182 (1985).
18. K. Wardle and R.T. Poole, Agricell Rep., **1**, 19 (1983).
19. K.E. Brainerd and L.H. Fuchigami, J. Am. Soc. Hort Sci., **106**, 515 (1981).