



In the Name of Allah
The Most Gracious
The Most Merciful
Whose Help We Solicit

**STUDIES ON TISSUE CULTURE
OF
RAUWOLFIA SERPENTINA (L.) BENTH. EX KURZ.**

**By
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**DEPARTMENT OF BOTANY
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STUDIES ON TISSUE CULTURE OF
RAUWOLFIA SERPENTINA (L.) BENTH. EX KURZ.

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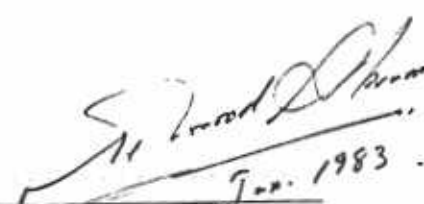
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Studies on Tissue Culture of *Rauwolfia serpentina* (L.) Benth. ex Kurz.

Abstract

The purpose of these investigations was to cultivate *Rauwolfia serpentina* callus tissue and to regulate differentiation for *in vitro* propagation. Leaf, stem and root explants of *R. serpentina* were cultured on Murashige and Skoog's (1962), White's modified (Root Culture) and Abou-Mandour (1977) media, with the supplementation of coconut milk, casein hydrolysate, yeast extract as organic nutrients and different concentrations of various plant growth regulators as 2,4-D, NAA, IAA, ABA, Kn, BAP, 2iP and adenine sulphate for callus formation and callus culture.

Morphogenetic studies were carried out and root and shoot differentiation was achieved. The differentiation of roots mostly occurred in stem callus and to a lesser degree in root callus. The *in vitro* differentiated roots were cultured successfully both for propagation of roots and for callus formation.

The shoot differentiation achieved in stem callus, led towards plantlets formation. The plantlets were transferred to soil where they flourished almost similar to field growing plants.

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INTRODUCTION

The term "Tissue Culture" is normally used as a blanket term to cover the culture of all plant parts ranging from a single cell to a group of cells, or an organ as a whole. Such technique provides a unique opportunity to study in depth the biochemical, biophysical and structural changes occurring during various phases of plant development (Thomas and Davey, 1975). The cultural techniques are used now as an alternative machinism for the asexual propagation of various plant species (Murashige, 1978).

Tissue culture has extended the area of involvement from the study of totipotency of cell (Steward *et al.*, 1958), to the study of synthesis of plant constituents in suspension cultures, the culture of meristem, pollen and protoplasts, single cell clones, plant tumors, biochemical mutants and somatic hydridization (Mitra, 1979).

The members of family Apocynaceae such as *Rauwolfia serpentina* are gaining much interest for tissue culture studies due to the presence of indole alkaloids. There have, however, been relatively few studies of these members concerning growth requirements of the tissue or plant regeneration in culture (Zenk *et al.*, 1977; Ohta and Yatazawa, 1979; Stockigt *et al.*, 1981). Ever since the early studies (Babcock and Carew, 1962; Mitra and Kaul, 1964), which are generally regarded as the first investigations on the *R. serpentina* culture, attention seems to have been focussed on

the chemical constituents of the cultures. Little is known of the growth requirements of long duration callus cultures and plant regeneration in *Rauwolfia* species. Therefore, the present investigations were undertaken with the object of extending our knowledge of tissue culture studies of *Rauwolfia serpentina* and achieving easy propagation of this wild member of the family Apocynaceae. The major source of roots of *R. serpentina* used as raw material for the extraction of isolated alkaloids, chiefly reserpine, rescinnamine, ajmaline and ajmalicine, presently are the wild plants.

Rauwolfia serpentina Benth. ex Kurz. grows wild in India, Bangla Desh, Sri Lanka, Burma, Malaya, Thailand and Java in moist deciduous forests ranging from sea level upto 1200 meters. *Rauwolfia* species cannot be easily cultivated due to various factors thus limiting their extensive culture.

Propagation of the plant by means of seeds has so far not been reasonably satisfactory. Studies on germination of viable seeds of *R. serpentina* showed a germination percentage from 10 to 75. (Mitra, 1976a). Moreover, the unfavourable climatic conditions, as varying degree of temperature and percentage of humidity prevailing during the months of July-August, cause shrivelling and desiccation of the endosperm. This arrests in turn the development of immature embryos and thus cause the formation of non-viable seeds in large number (Mitra, 1976a). Other means of propagation are by root stumps and by root, stem and leaf cuttings (George, 1964; Sitaram,

1978). Of these the only method which gives high percentage of successful plants is the propagation by root cuttings. However, the alkaloidal content of roots from plants raised by root cuttings is less than those raised from seed (Sitaram, 1978). Furthermore, the roots are not cherished for propagation by the growers due to their demand in the drug market.

This study would provide advantages expected from tissue culture systems over the conventional cultivation of whole plants. The detailed knowledge of cultural conditions for establishing the continuous cultures of *R. serpentina* would be helpful towards chemical studies and the production of useful *Rauwolfia* alkaloids.

The differentiation of roots in cultures and subsequent propagation of these roots would enable to relieve the reliance on wild plants. The differentiation of shoots and plant regeneration studies would help to overcome the difficulties presently met with the propagation of *R. serpentina* by seed.

From the literature it appears that there still exist many aspects to be explained in the tissue culturing of *R. serpentina*. There is a lack of detailed knowledge of cultural requirements and of plant regeneration. In the present investigations on *R. serpentina* tissue culture, a fairly detailed study was made of cultural requirements of *R. serpentina* tissue culture. Moreover special attention

was paid to the plant regeneration of *R. serpentina* through shoot bud differentiation in callus cultures.

REVIEW OF LITERATURE

I. Callus Culture

The parenchymatous cells become meristematic in the wounded parts of the plant. This phenomenon was noticed first by Haberlandt (1902) in parenchyma of potato. Moreover, this process might lead to the formation of a mass of undifferentiated cells as observed in other plant species. Callus can often be induced in many plant species when the wounding is combined with treatment of various synthetic and natural auxins and cytokinins. This newly formed callus mass, however, lacks internal organization into distinctive tissue systems. The tissue, nevertheless, has the potentiality for normal tissue differentiation and this potentiality is often expressed during healing of the wound.

Callus cultures are usually established from vegetative and reproductive organs of various plant species.

The first callus cultures were established by Gautheret (1939) from the roots of *Daucus carota*. Later, other workers reported callus cultures of carrot (Domanska and Aldona, 1976; Bender and Neuman, 1978a) and other angiosperms including a majority of medicinal plant species as *Mentha piperita* (Lin and Staba, 1961), *Digitalis lanata* and *D. purpurea* (Staba, 1962), *Ammi visnaga* (Kaul and Staba, 1965, 1967), *A. majus* (Chen et al., 1969) *Trigonella foenumgraecum*

(Subramaniam *et al.*, 1969), *Cassia senna* (Rai *et al.*, 1974), *Peganum harmala* (Nettleship and Slaytor, 1974), *Panax ginseng* (Jhang *et al.*, 1974; Chien and Staba, 1978), *Cannabis sativa* (Itokawa *et al.*, 1975), *Rheum palmatum* (Furuya *et al.*, 1975), *Swertia japonica* (Miura *et al.*, 1978), *Solanum laciniatum* (Hosoda and Yatazawa, 1979).

The callus cultures were also reported for various gymnosperms (Ramawat and Arya, 1979; Javeed *et al.*, 1980; Jansson and Bornman, 1981), ferns (Mehra and Pental, 1976), Mosses (Hahn and Miller, 1966), thalloid liverworts (Ilahi, 1972) and marine alga (Saga *et al.*, 1982).

The above list is not exhaustive, as the whole number of reported plant species with established callus cultures is too large to be reviewed here.

a. Callus formation on leaf explants

Vascular cambia (White, 1963), Cotyledons (Witham, 1968; Ikramullah, 1982), leaf mesophyll (Joshi and Ball, 1968), provascular tissue (Venketeswarn, 1962), protoplasts (Constabel *et al.*, 1973; Kartha *et al.*, 1974; Nagy and Maliga, 1976; Potrykus *et al.*, 1977; Shepard and Totten, 1977; Bajaj *et al.*, 1978; Simmonds *et al.*, 1979; Fowke *et al.*, 1981), bulb scales (Stimart and Ascher, 1978), petioles (Pillai and Hildebrandt, 1968a; Furuya *et al.*, 1972), fronds in aquatic plants (Chang and Pao-ling, 1976), mesocotyle and coleoptile segments in monocotyledons (Vuillaume and Deshayes, 1977) can produce callus

in leaf culture. The callus formation on leaf explants was reported for various plant species including *Hordeum vulgare* (Koblits and Saalbach, 1976) *Glycine max* (Evans *et al.*, 1976), *Crotolaria juncea* (Ramawat *et al.*, 1977), Sugarcane (*Saccharum officinarum* (Ji-shu, 1979), *Nigella sativa* (Chand and Roy, 1980), *Solanum nigrum* (Akram and Ilahi, 1982).

Mitra *et al.* (1965) obtained the leaf callus cultured of *Rauwolfia serpentina* by activating the phloem and parenchyma around the vascular region of leaf lamina. Callus culture from leaf of *R. serpentina* were also reported later by Mitra and Chaturvedi (1970).

b. Callus formation on stem explants

Vascular cambia (White, 1963), storage parenchyma (Skoog and Tsui, 1948; Steward *et al.*, 1952), pro-vascular tissue (Venketeswarm, 1962), and pith (Witham, 1968; Pillai and Hildebrandt, 1968a, Sadhu, 1974) can produce callus in stem culture. The callus formation on stem explants was reported for various plant species including sunflower (*Helianthus annuus*) (Henderson *et al.*, 1952), *Citrus grandis* (Chaturvedi *et al.*, 1974), *Choisya ternata* (Chenienx *et al.*, 1977), *Caralluma umbellata* (Regupathy *et al.*, 1977) *Matricaria chamomilla* (Szoke, *et al.*, 1977), *Crotolaria juncea* (Ramawat *et al.*, 1977), *Solanum nigrum* (Akram and Ilahi, 1982).

Callus cultures from stem of *Rauwolfia serpentina*

are also reported (Mitra and Kaul, 1964). Mitra *et al.* (1965) working on the induction of callus from stem segments and leaves of *R. serpentina* found that pith, phloem and cortex multiplied to form callus in stem segments. Perveen (1978) observed that callus was formed from stem segments of *R. serpentina*, when the basal (Knop's) medium was supplemented with 0.1 to 1.0 mg/l of indole acetic acid and naphthylene acetic acid; 0.1 to 0.5 mg/l of 2,4-dichlorophenoxyacetic acid and 0.5 to 1.0 mg/l of indole butyric acid. The supplementation of culture medium with 0.01 to 0.1 mg/l of kinetin, 10% coconut-milk and 500 mg/l of casein hydrolysate with above mentioned auxins had stimulatory effect on callus growth.

c. Callus formation on root explants

Storage parenchyma (Wiggans, 1954; Nitsch and Nitsch, 1956; Synno, 1965; Singh *et al.*, 1978), root pericycle (Goldacre *et al.*, 1962), root phloem (Fosket and Roberts, 1965), hypocotyles and radicles (Chalupa *et al.*, 1976; Amin, 1982) can produce callus in root culture. Callus formation on root explants was reported for various plant species including carrot (Syono, 1965; Yamaguchi and Nakajima, 1966; Sakano and Komamine, 1978), tomato (*Lycopersicon esculentum* (Norton and Boll, 1954), *Atropa belladonna* (West and Mika, 1957; Bhandary *et al.*, 1969), rice (*Oryza sativa*) (Yatazawa *et al.*, 1967; Cat and Uyen, 1976), *Solanum laciniatum* (Rerabek, 1967), *Vicia faba* (Grant and Fuller,

1968), *Panax ginseng* (Slepyon, 1971; Chang and Hsing, 1978), *Crotolaria juncea* (Ramawat *et al.*, 1977), *Matricaria chamomilla* (Sizoke, *et al.*, 1977) and Soy bean (*Glycine soja*) (Davidonis *et al.*, 1978).

Mitra and Kaul (1964) reported the formation of callus tissue from the radical (root) of an inoculated excised embryo of *Rauwolfia serpentina* growing on culture medium supplemented with 1.0 mg/l of 2,4-D and 10% coconut milk.

d. Callus formation on explants taken from reproductive organs

The cultures producing callus are also reported from various reproductive organs of the plant species such as entire flowers in *Matricaria chamomilla* (Sizoke, *et al.*, 1977); flower pedicels in *Solanum nigrum* (Akram and Ilahi, 1982); anthers in *Geranium* (Abo-El-Nil and Hildebrandt, 1971), *Chrysanthemum* (Watanabe *et al.*, 1972), Sweet cherry, strawberry (*Fragaria vesca*) (Degeida *et al.*, 1976), *Atropa belladonna*, *Nicotiana tabacum* (Bajaj *et al.*, 1978); Micro-and megasporophylls of Gymnosperms (Bonga, 1974); pollen in tomato (Sharp *et al.*, 1971), tobacco (Sharp *et al.*, 1971; Maliga *et al.*, 1973), *Pinus resinosa* (Bonga and Mcinnis, 1975); fruits in *Solanum nigrum* (Mandal and Gadgil, 1979; Akram and Ilahi, 1982); endosperm in *Ricinus communis* (Satsangi and Mohan Ram, 1965),

rice (Nakano *et al.*, 1975), *Petroselinum hortense* (Masuda *et al.*, 1977); ovules in cotton (Hsu and Stewart, 1976), *Citrus sinensis* (Kochba *et al.*, 1978) and embryos in rice (Maeda, 1971), *Euphorbia pulcherrima* (Nataraja, 1975), *Secale cereale* (Rybezynski, 1978, 1979), barley (*Hordeum vulgare*) (Granatek and Cockerline, 1978; Bayliss and Dunn, 1979) and *Pinus strobus* (Minocha, 1980).

II. Differentiation in Tissue Culture

The callus tissue remain undifferentiated or could be induced to form various plant organs under certain conditions. The primordia of such differentiating organs are formed *de novo* from a single or small groups of parenchymatous cells, which give rise to small meristematic regions.

The following types of differentiation are usually regognized in cell and tissue cultures.

a. Root differentiation

Root formation is the most common type of recognizable morphogenesis in cultures. Root differentiation was first reported from carrot cultures by Nobecourt (1939). Many other species including tobacco (Skoog and Miller, 1957), pea (Torrey, 1959), *Helianthus tuberosus* (Gautheret, 1969), Rice (Nakano and Maeda, 1974), *Populus euramericana* (Chalupa, 1974), *Glycine-max* (Evans *et al.*, 1976) Sugar-cane (Nadar and Heinz, 1977), Onion (*Allium cepa* var. *preliferum*) (Nandi

et al., 1977), *Pinus taeda*, *P. elliottii*, *P. rigida*, *P. radiata*, *P. sabiniana*, *P. virginiana*, *Pseudotsuga* sp. (Brown and Sommer, 1977), Ginseng (Chang and Hsing, 1978), *Phaseolus vulgaris* (Haissig, 1979), *Solanum nigrum* (Mandal and Gadgil, 1979) have been successfully studied for the induction of roots.

Perveen (1978) noticed the differentiation of roots in stem callus *R. serpentina* when 1.0 mg/l of naphthylene acetic acid was included in the Knop's medium.

b. Shoot bud differentiation

Adventitious buds arise during culturing from organized vegetative tissue via callus. They are often derived from single or a small group of cells and readily regenerate plants (Barlass, 1982).

The demonstration of the hormonal control of differentiation and organogenesis (Skoog and Miller, 1957) and proven totipotency of higher plant cells (Vasil and Hildebrandt, 1965) laid the foundation for the clonal propagation of plants through tissue culture techniques.

Development of plantlets through induced or regenerated shoot buds has been realized in *Convolvulus arvensis* (Bonnett and Torrey, 1965), *Trigonella foenumgraecum* (Subramaniam *et al.*, 1968), Rice (Nishi *et al.*, 1968), Sugarcane (Barba and Nickell, 1969), *Begonia rex* (Chlyah, 1972),

Chrysanthemum (Watanabe *et al.*, 1972), *Scopolia parvifolia* (Tabata *et al.*, 1972), *Euphorbia pulcherrima* (Nataraja *et al.*, 1973), *Gerbera jamesonii* (Pierik *et al.*, 1973), *Petunia inflata*, *P. hybrida* (Rao *et al.*, 1974), Poplar (Chalupa, 1974), *Helianthus annuus* (Sadhu, 1974), *Nigella damascena* (Raman and Greyson, 1974), *Medicago sativa* (Saunders and Singham, 1975), Grapevine CV. Cabernet-Sauvignon (Mullins and Srinivasan, 1976), *Picea abies*, *Pseudotsuga mensiesii*, (Chalupa, 1977) *Bacopa monnieri* (Thakur *et al.*, 1977), *Sisymbrium irio* (Pareek and Chandra, 1977) *Citrus sinensis* (Kochba *et al.*, 1978), *Lathyrus sativus* CV.LSD-6 (Mukhopadhyay and Bhojwani 1978; Banks *et al.*, 1979) and Carnation (*Dianthus caryophyllus*) (Roest and Bokelmann, 1981).

Mitra and Chaturvedi (1970) reported the differentiation of "organized bodies" (meristems) in leaf callus explants of *R. serpentina* growing on modified Murashige and Skoog's agar medium containing kinetin (0.5 mg/l), adenine/adenine sulphate (10 mg/l) and casein hydrolysate (500 mg/l) or yeast extract (200 mg/l) and 10% (v/v) of coconut milk. (Murashige and Skoog, 1962).

c. Embryoids formation

The most spectacular demonstration of the persistence of totipotency in the cells of higher plants is the regeneration of whole plant following the induction of embryoids in somatic-cell cultures. The embryoids were first obtained

in cells from tissue culture of carrot (Reinert, 1958 & 1959). The embryoida formation in cell and callus culture has later been confirmed with both cultivated and wild varieties of carrot (Kato and Takeuchi, 1963; Steward *et al.*, 1964; Halperin, 1966). Moreover, *in vitro* embryogenesis has also been reported in species from numerous angiosperms including *Raunculus seleratus* (Konar and Nataraja, 1965) *Dendrophoe falcata* (Johri and Bajaj, 1965) *Solanum melongena* (Yamada *et al.*, 1967), *Atropa belladonna* (Thomas and Street, 1970), *Coffea conephora* (Staritzky, 1970), *Brassica oleracea* (Lustinec and Horak, 1970), *Bromus inermis* (Constabel *et al.*, 1979) *Sinappis alba* (Bajaj and Bopp, 1972), *Nigella damascena* (Raman and Greyson, 1974), *Antirrhinum majus* (Sangwan and Harda, 1975), *Pimpinella anisum* (Hubur *et al.*, 1978), Sugarcane (Ji-Shu, 1979) and in various tissues of Cycads (Norstog and Rhamstine, 1967).

There is a fairly extensive literature on the *in vitro* embryogenesis but this will not be discussed further here since somatic embryogenesis was not considered in the present investigation, and comprehensive reviews are already available (Reinert, 1973; Narayanaswamy, 1977).

III. Organ Culture

Various plant organs have successfully been cultivated and the work concerning organ culture has briefly been reviewed in the following pages:

a. Root culture

The first successful root-cultures were those of Kotte (1922) and Robbins (1922), who independently observed the growth of excised root tips on mineral solutions supplemented with sugars, asparagine and peptone. White (1934) cultured excised root tips of *Lycopersicon esculentum* in a liquid medium containing inorganic salts, yeast extract and sucrose. Since then excised root cultures have been established from numerous plant species like Pea (Torrey, 1954), *Zea mays* (Maas, 1969), *Vicia faba* (Grant and Fuller, 1971), *Atropa belladonna* (Thomas and Street, 1972) Barley (Jacobson and Young, 1975), *Betula pubescens* (Momot *et al.*, 1975), Cereals (Mascarenhas *et al.*, 1975), *Larix sibirica* and *L. gmelinii* (Momot, 1977), *Raphanus sativus* (Evans and Verville, 1978), *Bryophyllum calycinum* (Robbins and Hervey, 1978) and ferns, (Kshirsagar and Mehta, 1979).

Mitra and Kaul (1964) reported the culture of excised seedling roots of *R. serpentina* by the supplementation of basal medium with 1.5 ppm of indole acetic acid and 45 ppm of adenine sulphate. Continuous cultures of roots of *R. serpentina* were maintained by Mitra (1968) on a modified White's medium.

b. Shoot buds culture

Robbins (1922) was the first one to culture the shoot apices. The shoot apices could be induced to form roots

in culture. Development of complete plants have been achieved from isolated shoot apical meristem of *Stellaria media* (White, 1933), *Tropaeolum majus*, *Lupinus albus* (Ball, 1946), *Sequoia sempervirens* (Ball, 1950), Dahlias (Morel and Martin, 1952), Potato (Kassanis, 1957), *Chrysanthemum cinerariaefolium* (Grewal and Sharma, 1978), *Geranium* (Pillai and Hildebrandt, 1968), *Asparagus* (Murashige *et al.*, 1972), *Saxifraga sp.*, *Begonia sp.*, *Gerbera jamesonii*, *Yucca sp.* (Murashige, 1974), *Hordeum vulgare* (Cheng and Smith, 1975) *Lolium multiflorum* (Dale, 1975), *Capsella bursapastoris*, *Senecio vulgaris* (Bapat and Rao, 1977), *Manihot esculenta* (Rey and Mroginski, 1978), *Cicer arietinum*, *Lens esculentum*, *Pisum sativum*, *Phaseolus aureus*, *P. mungo* (Bajaj and Dhanju, 1979), *Linum usitatissimum* (Lane, 1979) and Strawberry (Kantha *et al.*, 1980).

Shoot development and 'differentiation' in *R. serpentina* as reported by Perveen (1978) when the basal medium was supplemented with 0.5 mg/l naphthalene acetic acid and 10 mg/l kinetin; 0.01 mg/l and 0.05 mg/l indole acetic acid with 0.05 mg/l kinetin and with the addition of 10% coconut milk was actually the revival of growth in shoot bud already existing on the inoculated stem explant which in turn simultaneously produced both the callus and shoot.

Besides root and shoot apices, zygotic embryos (Narayanaswami and Norstog, 1964), pollen (Tulecke, 1965),

seeds (Staba *et al.*, 1965), flower buds (Blake, 1966) leaves (Steeves and Sussex, 1957) and bulbs (Heath and Hollies, 1965) have been successfully cultured *in vitro*.

IV. Suspension Culture

The earliest suspension of cultured plant cells capable of repeated subculture were developed from *Nicotiana tabacum* and *Tagetes erecta* (Muir, 1953). Suspension cultures composed of free cells and cell aggregates were also obtained from Pea (Torrey and Shigomura, 1957), Carrot (Kato and Takeuchi, 1963), Rice (Maeda, 1965), *Ranunculus sceleratus* (Konar and Natarajee, 1965), *Acer pseudoplatanus* (King *et al.*, 1973), *Phaseolus vulgaris* (Arinson and Boll, 1976), *Hevea brasiliensis* (Wilson *et al.*, 1976), *Choisya ternata* (Chenieux *et al.*, 1977), *Catharanthus roseus* (Kurz *et al.*, 1980; Kutney *et al.*, 1980), from normal and tumor tissues of *Picea glauca* (Reinert, 1956) and from haploid tissue of *Antirrhinum majus* (Melcher and Bergmann, 1959).

There have been many studies of suspension culture of plants for various reasons. But little attention has been paid to the suspension culture of *R. serpentina* except that such studies have been mainly focussed on alkaloid formation (Vollosovich *et al.*, 1977; Nikolaeva *et al.*, 1977; Stockigt *et al.*, 1981).

V. Pharmaceutical Tissue Culture

The recent and rapid advances in the development of plant tissue culture techniques have allowed the large scale cultivation of plant tissue and exploration of their biosynthetic capabilities. Rapidly growing suspension cultures of *Ruta graveolens* simultaneously produced volatile oils (Corduan and Reinhard, 1972), furanocoumarins (Austin and Brown, 1973), and alkaloids (Boulanger *et al.*, 1973).

In Japan *Phytolacca americana* suspension medium is being commercially used as a plant virus inhibitor (Liu, 1974). Diversified plant metabolites (Routien and Nickell, 1956; Metz and Lang, 1966; Robert and McCormick, 1973), diosgenin (Staba and Kaul, 1971), ginsenosides (Furuya and Ishii, 1972, 1973), glycyrrhizin (Tamaki *et al.*, 1973), L-dopa (Brain, 1974) and allergens (Staba and Shafiee, 1974) are being commercially produced from plant tissue culture.

Because of the potential economic importance, cultures from alkaloid containing plants have been most extensively developed. The plants reported for these studies include *Atropa belladonna* (West and Mika, 1957), *Datura sp.* (Chan and Staba, 1965; Hiraoka *et al.*, 1973), *Ipomoea violacea* (Staba and Laursen, 1966), *Peganum harmala* (Reinhard *et al.*, 1968), *Ruta graveolens* (Steck *et al.*, 1971), *Scopolia parviflora* (Tabata *et al.*, 1972), *Macleaya sp.* (Neumann and

Mueller, 1974), *Papaver somniferum*, *P. bracteatum* (Ikuta *et al.*, 1974) *Hyoscyamus niger* (Dhoot and Henshaw, 1977; Yamada and Hashimoto, 1982), *H. muticus* (Grewal *et al.*, 1979), *Catharanthus roseus* (Zenk *et al.*, 1977; Krueger and Carew, 1978) and *Ephedra gerardiana* Ramawat and Arya, 1979).

Cultural techniques were extensively used for the production of carboline (Veliky, 1972), indole (Nikolaeva and Vollosovich, 1972), edulinine, furoquinoline (Boulanger *et al.*, 1973), bioscoclaurine (Akasu *et al.*, 1976) and glycoalkaloids (Zacharius and Osman, 1977) and for the production of berberine (Furuya *et al.*, 1972) and hyoscyamine (Su, 1974).

Comprehensive reviews are available on pharmaceutical tissue culture (Staba, 1963; Staba *et al.*, 1965; Staba, 1969; Gamborg *et al.*, 1971; Turner, 1971; Zenk, 1977).

The aspect of pharmaceutical tissue culture has been studied for *R. serpentina* by many workers. Alkaloidal contents are reported for *R. serpentina* callus (Babcock and Carew, 1962; Yoshikawa *et al.*, 1973, 1977; Vollosovich *et al.*, 1977; Nikolaeva *et al.*, 1978; Ohta and Yatazawa, 1979) and suspension cultures (Nikolaeva and vollosovich, 1977; Stockigt *et al.*, 1981).

MATERIALS AND METHODS

Plant Materials

Experimental studies were carried out on *Rauwolfia serpentina* Benth. ex Kurz. (Family Apocynaceae). Various parts used for *in vitro* culture were obtained from plants growing at Medicinal Plant Farm of Pakistan Forest Institute, Peshawar; Department of Botany, University of Peshawar and P.C.S.I.R. Laboratories, Peshawar. Seeds harvested at Farm of Pakistan Forest Institute, were used for growing aseptic seedlings. Various parts of these aseptically grown seedlings were also used for culture.

Culture Methods1. Cleaning and sterilization of glassware

All the glassware was washed with detergent (Surf/Zip), then soaked in a mixture of potassium dichromate and concentrated sulphuric acid for about 15 minutes and washed in running tap water and rinsed with distilled water, it was autoclaved at 1 kg. per sq.cm pressure with 121 °C temperature for 20 minutes.

2. Sterilization of plant material

Stem, leaf and root explants taken from *R. serpentina* plants, were washed thoroughly with tap water, then surface sterilized by treating them with 1% solution of mercuric chloride respectively for 5-7, 7-10 and 3-7 minutes due to

the sensitive nature and moisture absorbing quality of various parts. They were then rinsed 3-5 times with sterile distilled water. The stem segments were cut aseptically. Sterile segments of leaf, stem and root were inoculated aseptically on the solidified agar media.

Seeds used for raising aseptic seedlings were sterilized with 1% solution of mercuric chloride containing 1% Tween 80 for 5 to 10 minutes and washed with sterilized water for 3 to 5 times.

The inoculation was done in a sterilized glass chamber supplied with ultra violet lights. The chamber was fumigated each time before using it for the inoculation with ethylene glycol and sprayed with ethyl alcohol.

3. Culture media

i) Murashige and Skoog's (MS) medium (1962) was employed for callus induction on stem and leaf explants and for further growth of induced calli. The composition of MS medium is as follows:

<u>Inorganic salts</u>			Concentration (mg/l)
Ammonium nitrate	NH_4	NO_3	1650
Potassium nitrate	KNO_3		1900
Magnesium sulphate	$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$		370
Potassium dihydrogen phosphate	KH_2	PO_4	170

<u>Micronutrients</u>		Concentration (mg/l)
Boric acid	$H_3 BO_3$	6.20
Manganese sulphate	$Mn SO_4 \cdot 4H_2 O$	22.30
Zinc sulphate	$Zn SO_4 \cdot 7H_2 O$	8.60
Sodium molybdate	$Na Mo O_4 \cdot 2H_2 O$	0.25
Cupric sulphate	$Cu SO_4 \cdot 5H_2 O$	0.025
Cobaltous chloride	$Co Cl_2 \cdot 6H_2 O$	0.025
Potassium iodide	KI	0.75

<u>Vitamins</u>		
Nicotinic acid		1.00
Thiamine-HCl		10.00
Pyridoxine-HCl		1.00
Myo - Inositol		100.0

Vitamins used for MS medium were according to B5 medium developed at the Prairie Regional Laboratory, Canada (Gamborg *et al.*, 1968).

Calcium chloride was used at 2.9 ml/l of medium from the stock solution made by dissolving 15 gms of $Ca Cl_2 \cdot 2H_2 O$ in 100 ml of distilled water according to Gamborg *et al.* (1968).

Sodium ethylenediamine-tetra-acetate (Na_2 EDTA) was added to the MS medium at the rate of 40 mg/l.

MS medium was supplemented with sucrose as carbohydrate source at a concentration of 2%.

ii) White's root culture medium (RC), as modified by Street (Thomas and Davey 1975) was employed for differentiation of roots from stem callus, for the growth of *in vitro* differentiated roots, for the induction of callus on these differentiated roots, and for the growth of root and stem calli. The composition of the RC medium is as under:

		Concentration (mg/l)
<u>Inorganic salts</u>		
Calcium nitrate	$\text{Ca} (\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	288.0
Magnesium sulphate	$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	370.0
Potassium chloride	KCl	65.0
Potassium nitrate	KNO_3	80.0
Sodium dihydrogen phosphate	$\text{Na H}_2 \text{PO}_4 \cdot 4\text{H}_2\text{O}$	21.50
Sodium sulphate	$\text{Na SO}_4 \cdot 10\text{H}_2\text{O}$	226.70
<u>Micronutrients</u>		
Boric acid	$\text{H}_3 \text{BO}_3$	1.50
Manganese chloride	$\text{Mn Cl}_2 \cdot 4\text{H}_2\text{O}$	6.0
Zinc sulphate	$\text{Zn SO}_4 \cdot 7\text{H}_2\text{O}$	2.65
Cupric sulphate	$\text{Cu SO}_4 \cdot 5\text{H}_2\text{O}$	0.02
Molybdic acid	$\text{H}_2 \text{Mo O}_4$	0.0017
Potassium iodide	KI	0.75

<u>Vitamins</u>	Concentration (mg/l)
Glycine	3.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.1
Thiamine-HCl	0.1

Ferric ethylenediamine-tetra-acetate (Fe EDTA) was used as 7.84 mg/l. The stock solution was prepared by dissolving 740 mg of Na_2EDTA and 324 mg of anhydrous Ferric chloride in 100 ml of distilled water. FeEDTA was used as 1 ml/l from the stock solution.

Sucrose was added as 1.5% to RC medium

Extra Nitrogen Source (ENS)

The stock solution was made by dissolving 0.32 gm of calcium nitrate $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 10 gm of Potassium nitrate in 100 ml of distilled water. This solution was added as ENS and at the rate of 10 ml/l to RC medium when mentioned. It was used when required to increase the nitrogen level of RC medium.

iii) Abou-Mandour Medium (1977) was used for the growth of leaf, stem and root calli. The composition of AM medium is as under:

<u>Inorganic salts</u>	Concentration (mg/l)
Ammonium nitrate $\text{NH}_4 \text{NO}_3$	650.0

		Concentration (mg/l)
Potassium nitrate	KNO_3	1080.0
Calcium nitrate	$\text{Ca} (\text{NO}_3)_2 \cdot \text{H}_2\text{O}$	320.0
Sodium sulphate	$\text{Na}_2 \text{SO}_4$	900.0
Magnesium sulphate	$\text{Mg} \text{SO}_4 \cdot 7\text{H}_2\text{O}$	265.0
Potassium chloride	KCl	65.0
Sodium dihydrogen phosphate	$\text{Na} \text{H}_2 \text{PO}_4 \cdot \text{H}_2\text{O}$	45.0
disSodium hydrogen phosphate	$\text{Na}_2 \text{HPO}_4 \cdot 12\text{H}_2\text{O}$	150.0
<u>Micronutrients</u>		
Manganese sulphate	$\text{Mn} \text{SO}_4 \cdot \text{H}_2\text{O}$	9.0
Zinc sulphate	$\text{Zn} \text{SO}_4 \cdot 7\text{H}_2\text{O}$	6.0
Boric acid	$\text{H}_3 \text{BO}_3$	6.3
Potassium iodide	KI	0.75
Cobaltous chloride	$\text{Co} \text{Cl}_2 \cdot 6\text{H}_2\text{O}$	0.25
Sodium molybdate	$\text{Na} \text{Mo} \text{O}_4 \cdot 2\text{H}_2\text{O}$	0.25
<u>Vitamins</u>		
Thiamine-HCl		0.1
Pyridoxine-HCl		0.5
Nicotinic acid		0.5
Glycine		2.0
Meso - Inositol		10.0
Folic acid		0.3

FeEDTA was used after RC medium (Thomas and Davey, 1975).

Sucrose was added as 3% to AM medium.

AM medium was always supplemented with following growth regulators and in the concentrations given against them, unless otherwise mentioned.

	Concentration (mg/l)
6-Benzylaminopurine (BAP)	0.1
Adenine sulphate (AS)	4.0
1-Napthylacetic acid (NAA)	1.0
Kinetin (Kn)	0.3
2,4-Dichlorophenoxyacetic acid (2,4-D)	6.0

All the above media were solidified with 1.0% Difco-bacto agar. Agar was omitted, for preparing suspension and liquid culture media. Suspension media were used on orbital shakers. Liquid media were not used on shakers, instead the inoculated materials in the, were supported on filter paper.

pH of the various media was adjusted with 1N Na OH and 0.1N HCl and is mentioned at appropriate places in the results.

iv) Knop's solution (Gautheret, 1952) was employed for watering of potted plantlets obtained in *in vitro* cultures.

It had the following composition:-

		Concentration (mg/l)
Calcium nitrate	$\text{Ca} (\text{NO}_3)_2 \cdot \text{H}_2\text{O}$	1000.0
Potassium nitrate	KNO_3	250.0
Magnesium sulphate	$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	250.0
Monopotassium dihydrogen phosphate	$\text{KH}_2 \text{PO}_4$	250.0

For trace elements, five drops of Berthelot's solution were added to each litre of Knop's solution. Berthelot's solution had the following composition:-

		Concentration (mg/l)
Ferrous sulphate	$\text{Fe}(\text{SO}_4)_3$	50,000.0
Manganese sulphate	Mn SO_4	2,000.0
Potassium iodide	KI	500.0
Nickel chloride	Ni Cl_2	50.0
Zinc sulphate	Zn SO_4	100.0
Copper sulphate	Cu SO_4	50.0
Boric acid	$\text{H}_3 \text{BO}_3$	50.0
Molybdic acid	$\text{H}_3 \text{MoO}_4$	500.0
Sulphuric acid	$\text{N}_2 \text{SO}_4$	1.0 ml

Sterilized distilled water was used throughout this investigation for making media, stock solutions and adjusting concentrations. All the stock solutions were stored at -1°C

to $+3^{\circ}\text{C}$., except vitamins, which were stored at -18°C .

4. Sterilization of media

For culture studies in general, culture tubes 25 x 150 mm size and 100 and 250 ml capacity Erlenmeyer flasks were poured in with 20, 50 and 100 ml of the medium. Suspension medium was poured in as 10 ml per 250 ml flasks. Lint free long staple cotton was used for plugging the culture flasks. The mouth of the flasks were sealed with tin foil. The media were sterilized within the culture flasks and tubes by autoclaving at 1 kg per sq. cm pressure and 121°C temperature for 5 to 10 minutes (Meynell and Meynell, 1970).

5. Organic nutrients

i) The coconut milk (CM) used in the media was prepared by collecting the liquid endosperm from the unripe coconuts. The CM collected from several nuts was heated to 80°C by stirring and then filtered and stored in the freezer. CM when used was 100 ml/l in the medium unless otherwise stated.

ii) Caesin hydrolysate (CH), when used in MS and RC media, was at the rate of 500 to 1000 mg/l. CH was added to AM medium as 1000 mg/l.

iii) Yeast extract (YE) when used in culture media was in concentration of 2000 mg/l unless otherwise stated.

iv) Biotin (Vitamin H) was used at 10 mg/l concentration.

6. Growth regulators

The following plant growth regulators were used in the solution form. Concentrations used in the experimental work are mentioned at appropriate places in the results. The growth regulators were added to the culture medium before sterilization.

Abscisic acid	$C_{15} H_{20} O_4$	(ABA)
Adenine sulphate	$(C_5H_5N_5)_2 \cdot H_2SO_4 \cdot H_2O$	(AS)
N^6 -Benzyladenine		
(6-Benzylaminopurine)	$C_{12} H_{11} N_5$	(BAP)
2, 4-Dichlorophenoxyacetic acid	$Cl_2 C_6 H_3 OCH_2COOH$	(2,4-D)
6-dimethylaminopurine	$C_7 H_9 N_5$	(2iP)
6-Furfurylaminopurine		
(Kinetin)	$C_{10} H_9 N_5O$	(Kn)
Gibberellic acid	$C_{19} H_{22} O_6$	(GA ₃)
3-Indoleacetic acid	$C_{10} H_9 NO_2$	(IAA)
3-Indolebutyric acid	$C_{12} H_{13} NO_2$	(IBA)
1-Naphthylacetic acid ()	$C_{10} H_7 CH_2 COOH$	(NAA)

7. Special chemicals

Sodium diethyldithiocarbamate (SDDC) was used when

required, at a concentration of 250 mg/l in the culture media. It was used for the control of browning of callus.

8. Growth conditions

The experiments were carried out in controlled 16 hours cycled fluorescent light cooled incubators (except otherwise mentioned). The experiments were carried out at $28 \pm 1^{\circ}\text{C}$ temperature throughout this investigation except otherwise mentioned.

Orbital shaker with controlled illumination, temperature and speed adjusted to 60 rpm, was used for growth of suspension cultures.

Both callus and suspension cultures were subcultured at regular intervals of 20 to 60 day incubation period, depending upon the requirement of individual experiments.

9. Aseptic seedlings

Aseptic seedlings were raised for the excision of leaf and root explants used in investigations on callus formation and callus growth. Seeds were surface sterilized and their testa were manually cracked under aseptic conditions. They were inoculated on solidified RC medium supplemented with 1.0 mg/l 2,4-D in sterilized culture tubes. The cultures were placed for seed germination at a temperature of 30°C in dark.

After germination of seeds by third week, the cultures were left for a further one week in dark and thereafter were placed under 16:8 hours light: dark regime. The temperature was lowered to 28°C. After 5th week of seed germination (Fig. 1A) the cultures were provided with 10% CM. The seedlings after growth of 8 weeks on CM were given with half strength (0.5 mg/l) 2,4-D for a further 3 weeks. The 2,4-D was excluded from the culture medium afterward (Fig. 1B). The age of seedlings at the time of excision of leaf and root explants is mentioned under respective experiments.

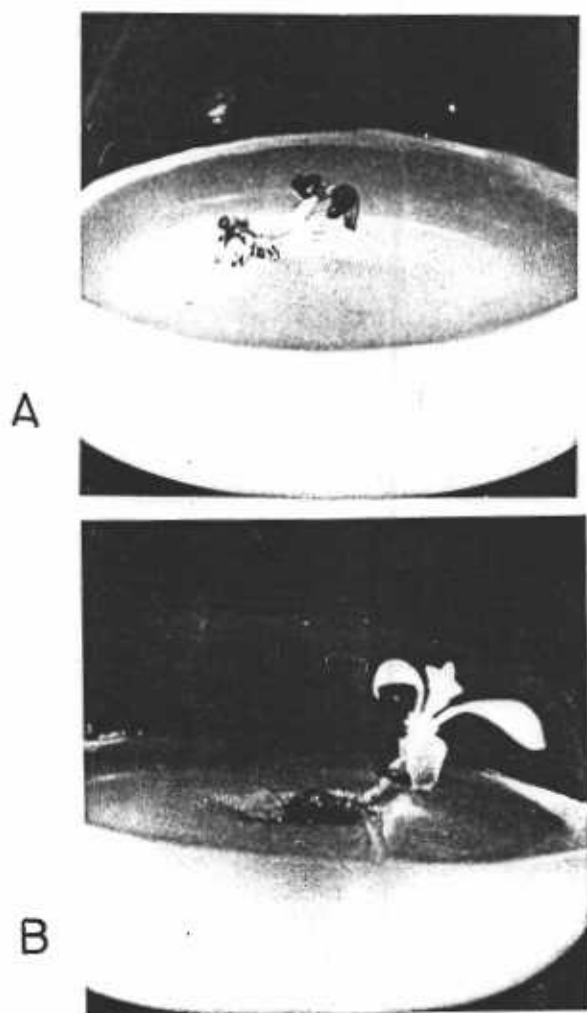


Fig. 1. Aseptically raised seedling of *Rauwolfia serpentina*, growing on White's Root Culture (RC) medium supplemented with (A) 1.0 mg/l 2,4-D (35 days old), (B) 100 ml/l CM. (140 days old).

RESULTS

I. CALLUS FORMATION AND CALLUS GROWTH

It is evident from the literature on plant tissue culture that the callus tissues are usually initiated from organ explants under the influence of varying concentrations of growth regulators (Thomas and Davey, 1975). Therefore, the investigations were started by examining the effects of various plant growth regulators on callus formation and callus growth. Preliminary studies were carried out to find the optimal conditions of callus formation including the type of regulators and their effective concentrations.

Following growth regulators were used:

- | | | | |
|-----------------|------|--------------------------------|---------|
| (1) auxins: | I. | 1-Naphthylacetic acid | (NAA) |
| | II. | 2,4-Dichlorophenoxyacetic acid | (2,4-D) |
| (2) cytokinins: | I. | Kinetin | (Kn) |
| | II. | Benzylaminopurine | (BAP) |
| | III. | Adenine sulphate | (AS) |

These regulators along with CM, YE and CH were tested against leaf, stem and root explants of *R. serpentina*.

The results were grouped into the following three categories:

- (1) Formation of callus by leaf explants and its

, further growth.

(2) Formation of callus by stem explants and its further growth.

(3) Formation of callus by root explants and its further growth.

1-1. Formation of Callus by Leaf Explants and its Further growth.

Leaf explants of *R. serpentina* were collected from actively field growing plants in early summer. Leaf explants taken from seedlings, aseptically raised in the laboratory, were also cultured. As is generally reported that callus is initiated on leaf explants towards cut surface of petiole; by the rupture of petiole and as callus swellings on lower midrib portion, therefore, the leaf explants in the present studies included these parts. MS medium was used for leaf explant cultures unless otherwise mentioned. CM when used was in the concentration of 100 ml/l of medium. CH where used was 500 mg/l unless otherwise mentioned. The pH of the medium was adjusted to 5.8.

(1) Culture of Leaf Explants from Field Grown Plants.

a) Effect of 2,4-D alone or with Kn in absence or presence of CM or CH.

The leaf explants were cultured on medium having 0.5 mg/l 2,4-D with or without CM.

The explants cultured without CM, initiated callus after 8 weeks. This callus was cultured on fresh medium containing CH. The auxin 2,4-D was replaced by 1.0 mg/l each of NAA and Kn. The callus grew for 4 weeks, then turned brown and ceased growth.

The leaf explants cultured with CM in the medium initiated callus after 6 weeks. The callus started browning within one week of initiation. When transferred to fresh medium containing CH and 2,4-D in combination with 1.0 mg/l Kn or CH with 1.0 mg/l NAA, the callus failed to grow within 4 weeks.

The leaf explants were cultured in medium having 1.0 mg/l 2,4-D and with or without either CM or 1000 mg/l CH.

The explants cultured without CM or CH exhibited swellings at the midrib and on petiolar portion after 5 weeks. The callus was observed on the ruptured swellings after 8 weeks without any further apparent growth.

The explants cultured with CM initiated callus after 8 weeks. The callus started browning within one week of formation.

The callus was, recultured on fresh medium containing CH. Either 2,4-D in combination with 1.0 mg/l Kn or only 1.0 mg/l NAA was added to the medium. These cultures showed no growth in 4 weeks.

The explants cultured with 1000 mg/l CH produced some callus after 12 weeks (Fig. 2B). Replacing CH with CM did not improve the callus growth in another 4 weeks.

b) Effect of NAA alone or with BAP or Kn in absence or presence of CM or CH.

The leaf explants were cultured on medium having 1.0 mg/l NAA. The medium was with or without CM.

The explants cultured without CM, produced no appreciable amount of callus within 12 to 13 weeks period (Fig. 2A). The little callus which formed during this period turned brown and stopped growing.

The explants cultured with CM in the media only exhibited some swellings within five weeks. These explants were cultured on fresh medium containing CH in place of CM and 0.5 or 1.0 mg/l Kn with available NAA. No callus formation occurred upto 4th week. These leaf explants, were recultured on fresh medium containing CH and 0.5 mg/l BAP with available NAA. Again, there was no callus formation upto 15 weeks.

Leaf explants were cultured on the medium with 1.0 mg/l NAA and 0.5 mg/l BAP and with either CM or CH or with neither of them.

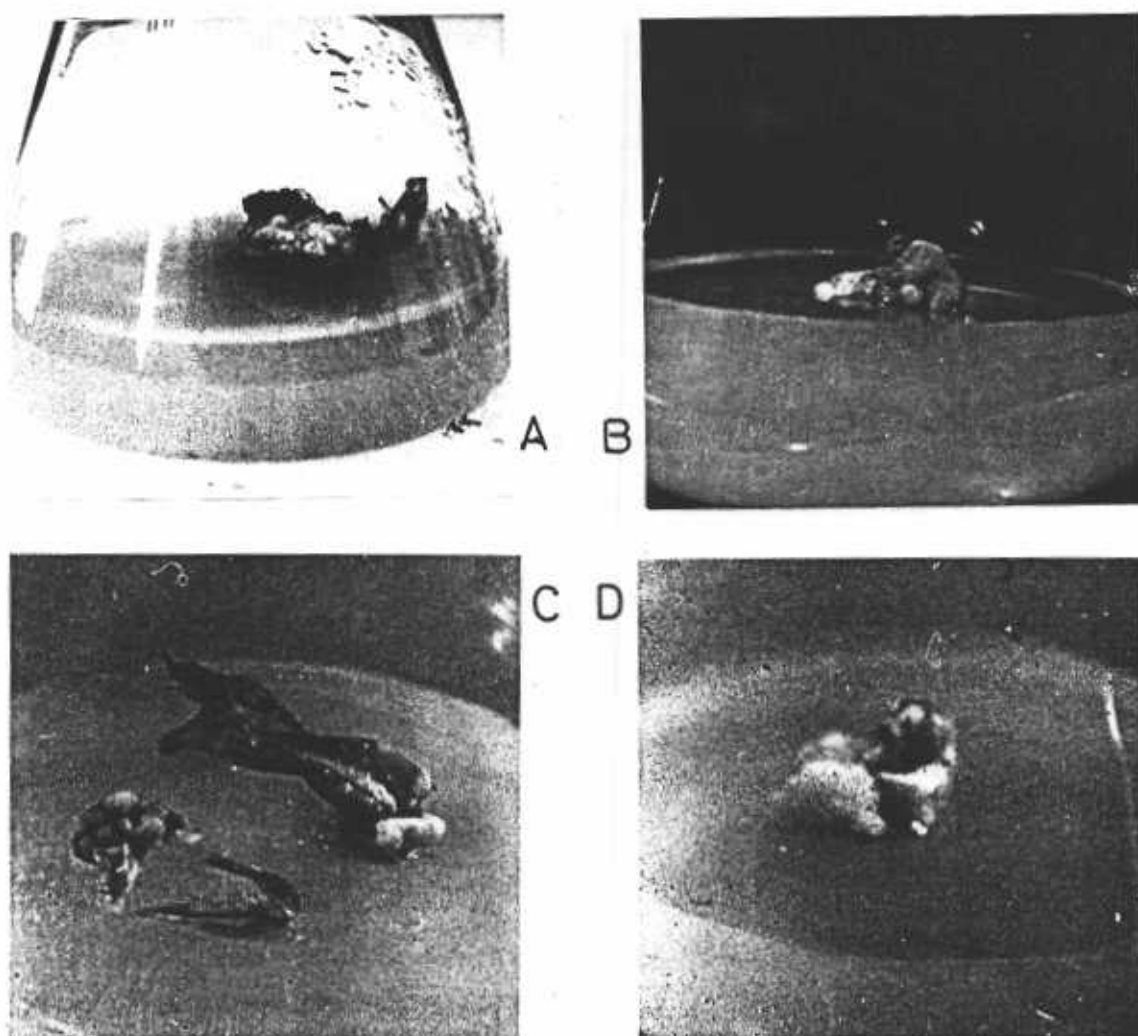


Fig. 2. Callus formation on leaf explants taken from field growing plants of *Rauwolfia serpentina*, cultured on MS medium with:

- (A) 1.0 mg/l NAA
- (B) 1.0 mg/l 2,4-D and 1000 mg/l CH
- (C) 1.0 mg/l NAA, 10.0 mg/l Kn and 100 ml/l CM
- (D) Explants taken from aseptic seedlings, cultured with similar treatment and for same duration (4 weeks) as for (C).

In explants cultured without CM or CH, no callus formation occurred upto 6 weeks. In an attempt to stimulate callus formation, these explants were then cultured on RC medium containing CH. The medium contained either similar concentration of regulators or the concentration was lowered to 0.1 mg/l for both NAA and BAP. The medium with these supplements failed to stimulate callus formation upto 6 weeks.

In explants cultured with either CM or CH no callus formation was seen upto 8 weeks.

c) Effect of transfer of NAA and BAP leaf explants to 2,4-D medium.

The leaf explants were cultured on medium containing CM and 1.0 mg/l NAA and 0.5 mg/l BAP for 8 weeks. The explants were, therefore, transferred to medium containing CM and 0.1 mg/l 2,4-D. The callus formation occurred after 7 weeks. The callus was of green colour. Transferring the callus to 1.0 mg/l 2,4-D did not improve the callus growth in another 6 weeks.

Leaf explants were then cultured on medium containing CM, 1.0 mg/l NAA and 10.0 mg/l Kn. A slight swelling was noticed by after 4 weeks. At this stage the light automation control of incubators broke down. Therefore, these cultures were subjected to 24 hours light. The explants under continuous light for 4 consecutive weeks initiated callus (Fig. 2C).

- d) Effect of NAA alone on with BAP in absence or presence of CM or CH on RC medium.

The leaf explants were cultured with 0.5 mg/l NAA on RC medium which did not contain, CM or CH. A slight swelling of the midrib portion was noticed after 5 days but no callus formation could be observed upto 6 weeks.

The explants when cultured on RC medium containing 1.0 mg/l NAA and 0.5 mg/l BAP and supplemented with either CH or CM, did not form callus in 8 weeks. These cultures with CM were later transferred to MS medium having 0.1 mg/l 2,4-D in place of NAA and BAP. The change of medium and regulators also failed to effect callus formation.

(2) Culture of Leaf Explants from Aseptically Grown Seedlings.

A. Under Varied Light Conditions:

- a) Effect of 2,4-D in the presence of CM.

Explants cultured on medium containing CM and 1.0 mg/l 2,4-D did not form callus during the first 4 weeks. Then the defect in incubator light system forced to grow the cultures in continuous light. The callus was observed during next 25 days on these explants under continuous light (Fig. 3A).

- b) Effect of NAA with Kn or BAP in presence of CM.

Explants cultured on medium containing CM and 1.0 mg/l

A



B



Fig. 3. Leaf callus growing on MS medium supplemented with 100 ml/l CM and (A) 1.0 mg/l 2,4-D
(B) 1.0 mg/l NAA and 10.0 mg/l Kn.

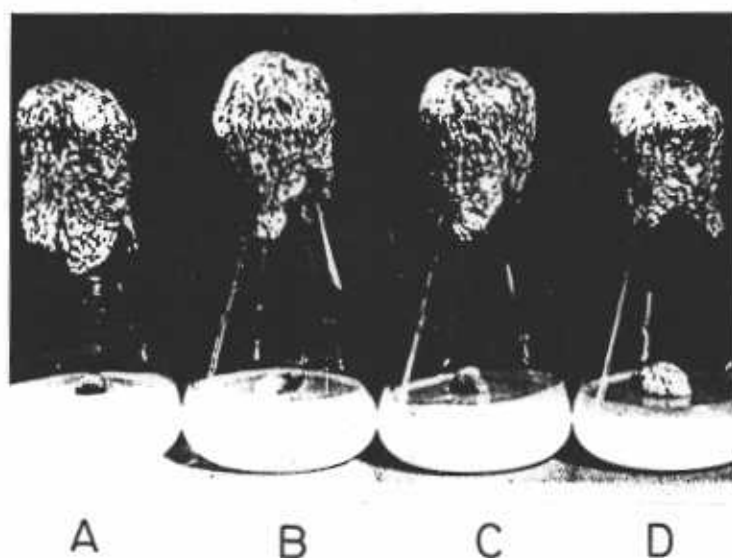


Fig. 4. Leaf callus growth in 4 weeks on MS medium supplemented with 100 ml/l CM and
(A) 1.5 mg/l NAA and 15 mg/l Kn
(B) 1.0 mg/l NAA and 10 mg/l BAP, and on Abou-Mandour (AM) medium supplemented with 1000 mg/l CH and
(C) 1 mg/l NAA and 10 mg/l Kn and
(D) standard concentration of AM regulators.

NAA and 10.0 mg/l Kn showed parallel behaviour to those with 2,4-D when exposed to continuous light after 4 weeks. Callus formed after 20 days in continuous light (Fig. 2D and table-1). This leaf callus grew under continuous light for a further period of 10 weeks (Fig. 3B) and developed some green colour. When these cultures were transferred to 16 hours light regime, the callus, after two weeks, developed greenish-white colour and appeared friable. By the end of 16th week cessation of growth occurred. When transferred to continuous light the resumption of growth did not take place but rather the desiccation ensued after two weeks.

In an effort to successfully culture the callus, its pieces were grown on medium containing 1.0 mg/l NAA and 10.0 mg/l BAP as well as with 1.5 mg/l NAA and 15.0 mg/l Kn. After one week the callus apparently regained moisture and by next week exhibited some growth on both variations of regulators. In another 4 weeks, however, the callus exhibited no appreciable growth (Figs. 4A and 4B).

A. Standard Light Conditions:

Effect of AM medium on callus growth.

The leaf callus cultures growing on MS medium containing 1.0 mg/l NAA and 10.0 mg/l Kn or BAP were transferred to AM medium. One batch of AM medium was modified by adding 1 mg/l NAA and 10 mg/l Kn instead of standard concentration of

Table 1: Callus formation on leaf explants of *Rauwolfia serpentina* on MS and White's Root Culture (RC) media with different treatments of growth regulators, 100 ml/1 Coconut milk (CM) and 500 mg/1 Caesin hydrolysate (CH) unless otherwise stated. The explants were incubated under standard light condition unless otherwise stated.

Explants origin	Treatment		Callus		Remarks
	Medium	Growth regulators (mg/l)	Nutrient	Formation	
Field Plants	MS	2,4-D	0.1	CM	49
			0.5	CM	56
			0.5	-	56
			1.0	CM	56
			1.0	-	56
			1.0	CH*	84
	NAA	0.5	-	-	-
		1.0	CM	-	-
		1.0	-	+	84
	NAA+Kn	1.0+0.5	CH	-	-
		1.0+1.0	CH	-	-
		1.0+10.0	CM	++	28
					Light continuous

Table 1: continued

Explants origin	Treatment		Callus		Remarks
	Medium	Growth regulators (mg/l)	Nutrient	Formation	Initiation period (days)
RC		NAA+BAP	1.0+0.5	CM	-
			1.0+0.5	-	-
			1.0+0.5	CH	-
			1.0+0.1	CH	-
			1.0+0.5	CH	-
			1.0+0.5	CM	-
Aseptic	MS	2,4-D	1.0	CM	25 Light continuous
seedlings		NAA+Kn	1.0+10.0	CM	20 Light continuous

Legend

- no callus.
- + fair callus.
- ++ good callus.
- +++ copious callus.

* concentration 1000 mg/l

regulators. After one week the callus apparently regained moisture and by next week exhibited good growth on both the standard and modified AM media. The growth was better than for the same callus on MS medium (Figs. 4A-4B). In another 4 weeks, the callus showed appreciable amount of growth on only the standard AM medium.

The callus exhibited active growth for next 13 weeks (Table-2). The shape of callus was like inverted cups. In another 32 weeks, the growth gradually slowed down to a minimum and ultimately ceased by 69th week.

1-2. Formation of Callus by Stem Explants and its further growth.

Twenty five to 30 mm stem pieces of *R. serpentina* taken from actively growing plants were cultured on MS medium. CH was used in the concentration of 500 mg/l unless otherwise mentioned and CM when added was always 100 ml/l of the medium. The concentration of YE is given in the respective experiments. Biotin and SDDC when used was 10.0 mg/l and 250 mg/l respectively. The pH of the media was adjusted to 5.8.

a) Effect of 2,4-D alone or with Kn in absence or presence of CH.

The stem explants were cultured on medium containing 1.0 mg/l 2,4-D. After 4 weeks callus was initiated (Table-3), which continued its growth till 13th week after that the growth stopped. When sub-cultured on 0.5 mg/l

Table 2: Growth in leaf callus of *Rawolfia serpentina* on MS and Abou-Mandour (AM) media with different treatments of growth regulators, 100 ml/l CM and 500 mg/l CH unless otherwise stated. The callus was cultured under standard light condition unless otherwise stated.

Callus origin	Treatment		Callus		Remarks
	Medium	Growth regulators (mg/l)	Nutrient	Growth	
Field Plants	MS	2,4-D 0.1	CM	-	-
		1.0	-	-	-
		1.0	CM	+	6
		1.0	CH*	-	-
		2,4-D+Kn 1.0+1.0	CH	-	-
		NAA 1.0	-	-	-
		1.0	CH	-	-
		NAA+Kn 1.0+1.0	CH	+	4
		1.0+10.0	CM	+	6
		1.5+15.0	CM	+	6
AM		NAA+BAP 1.0+10.0	CM	+	6
		NAA+Kn 1.0+10.0	CH*	++	2

Light continuous/

Table continued

Callus origin	Treatment		Callus		Remarks
	Medium	Growth regulator (mg/l)	Nutrient	Growth	
Aseptic seedlings	MS	NAA+Kn	1.0+10.0	CM	+++
			1.0+10.0	CM	+++
		2,4-D	1.0	CM	+++
	AM	Standard**		CH*	+++
					10
					6
					9
					13

Legend:-

- no growth.
- + fair growth.
- ++ good growth.
- +++ copious growth.
- * concentration 1000 mg/l
- ** 1 NAA+0.1 BAP+0.3Kn+4AS+62,4-D (mg/l)
- / light for 24 hours without dark period.
- // light for 16 hours/8 hours dark cycled.

2,4-D, the callus grew further for 8 weeks and then the growth totally ceased. Similar observations were noted when the callus was sub-cultured on auxin-less medium.

The 2,4-D callus when sub-cultured with CH and either 1.0 mg/l 2,4-D alone or combined with Kn, it grew for 4 weeks and then the growth slowed down.

b) Effect of NAA with Kn and YE

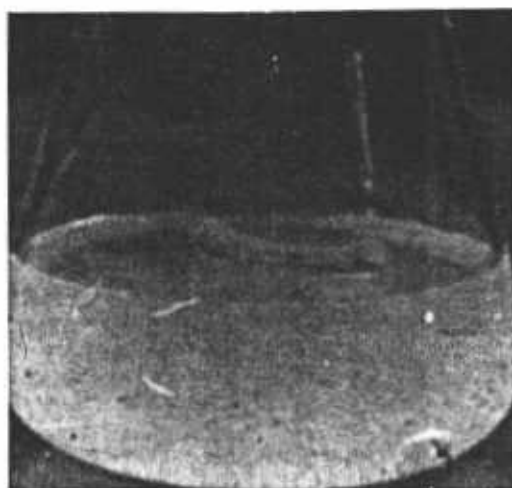
The stem explants were cultured on medium containing 0.5 mg/l NAA, 0.2 mg/l Kn. and 1000 mg/l YE. The explants showed some swelling without any definite callus formation in 4 weeks (Fig. 5B). When these explants were transferred to AM medium there was just a hint of callus formation in 6 weeks.

c) Effect of NAA alone or with Kn or BAP in the absence or presence of CH or CM.

Stem explants cultured on medium containing 1.0 mg/l NAA initiated callus after 6 weeks which continued its growth for another 11 weeks. When sub-cultured on medium containing 0.5 mg/l NAA in combination with either 0.5 or 1.0 mg/l Kn, the callus pieces grew for 8 weeks. Addition of CM to the medium extended callus growth for another 4 weeks.

The callus was later sub-cultured on fresh medium

A



B



Fig. 5. Callus formation in 2 weeks on stem explants of *Rauwolfia serpentina* cultured on MS medium with
 (A) 1.0 mg/l NAA, 0.5 mg/l Kn and 1000 mg/l CH
 (B) 0.5 mg/l NAA, 0.2 mg/l Kn and 1000 mg/l YE.

(B) treatment as in (A) except that 2 mg/l Kn was added.

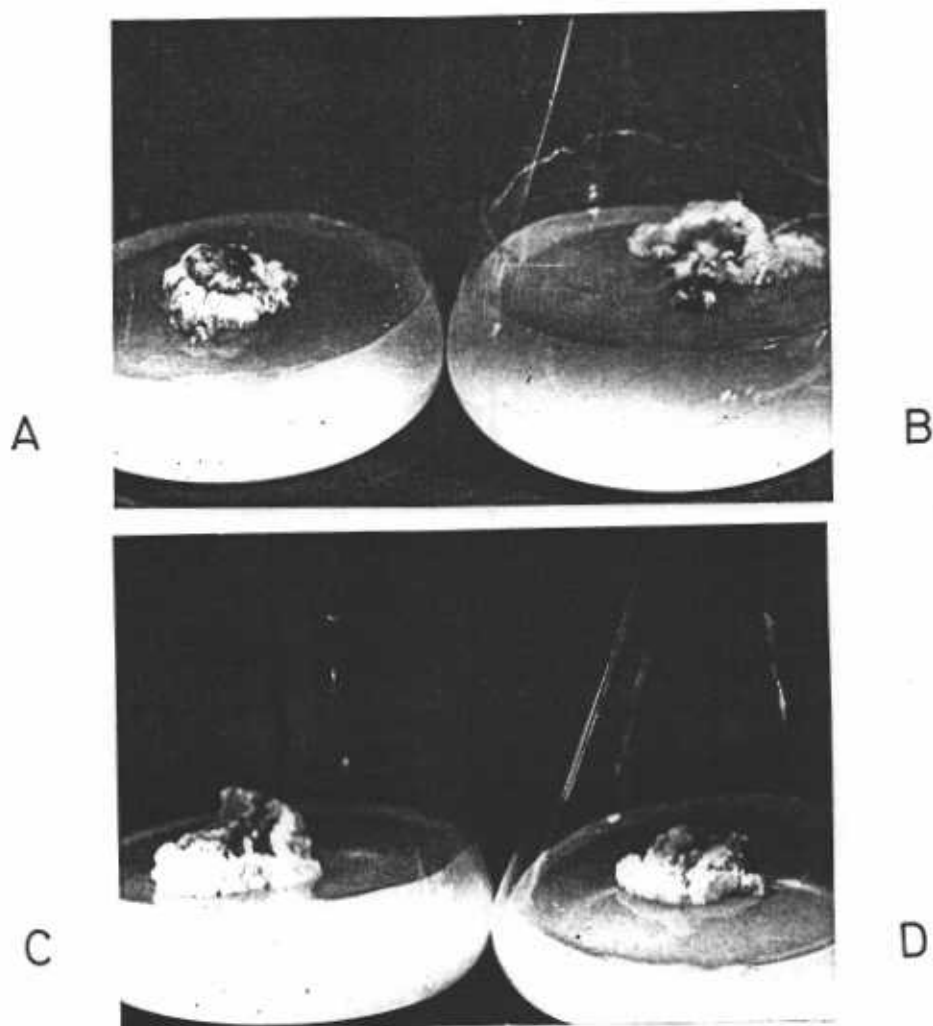


Fig. 6. Stem callus growing on MS medium supplemented with 100 ml/l CM and ;
 (A) 1.0 mg/l 2,4-D
 (B) 1.0 mg/l NAA and 0.5 mg/l Kn, and ;
 (C) On Abou-Mandour medium supplemented with 1000 mg/l CH and standard regulators
 (D) Treatment as in (C) except that 2 mg/l NAA was added.

Table 3: Callus formation on stem explants of *Rawolfia serpentina* on MS and Abou-Mandour (AM) media with different treatments of growth regulators, 1000 mg/l CH and 1000 mg/l yeast extract (YE).

Treatments			Callus	
Medium	Growth regulators (mg/l)	Nutrient	Formation	Initiation period (days)
MS	2,4-D 1.0	-	++	28
	NAA 1.0	-	++	42
	NAA+Kn 0.5+0.2	YE	-	-
	1.0+0.5	CH	+	13
AM	Standard*	CH	+	42

Legend:-

- no callus.

+ fair callus.

++ good callus.

* 1NAA+0.1BAP+0.3Kn+4AS+6 2,4-D (mg/l).

without CM but with 0.1, 0.5 and 1.0 mg/l NAA and similar concentrations of BAP. The callus growth was better with 0.5 mg/l NAA with either of these BAP concentrations till 5th week.

In order to sustain the continuous growth of the callus further sub-cultures were made with various concentrations of NAA and BAP and their combinations were attempted:

i) When sub-cultured to fresh medium containing 0.5 mg/l NAA and either 0.1, 0.5, 1.0, 1.5, 2.0 mg/l BAP, the callus showed somewhat better growth after 2 weeks with 0.5 mg/l NAA and 1.0 mg/l BAP. The growth was active for another 3 weeks and then slowed down.

ii) These callus cultures when sub-cultured on RC medium containing 0.1 mg/l NAA and BAP each, the callus grew for another 12 weeks (Fig. 8A). The callus was compact and milky-white in appearance.

iii) The callus was further sub-cultured on medium containing 0.1 mg/l NAA and BAP and either with or without CM. Callus continued its growth and, after 4 weeks, showed patches of green pigmentation and became more friable and nodular. The callus growth was similar in cultures with or without CM but after 6 weeks callus with CM was changing from nodular to loose spongy mass. After 10 weeks, the growth further improved

in cultures growing with CM (Figs. 8B and 8C). These cultures continued to form spongy callus in contrast to more compact in cultures growing without CM. The cultures without CM later on developed pink and hyaline areas while those with CM became offwhite in colour which persisted for another 12 weeks.

Pieces from pink, green, hyaline and white areas of callus in cultures growing without CM were isolated and subcultured on media with the same concentrations of NAA and BAP with or without CM. All the colours had the tendency of mixing and reappearing. Pink colour was noted as changing to pinkish green, green to greenish hyaline and vice versa and hyaline areas appeared in white coloured callus.

The milky-white coloured callus found in the cultures without CM (Fig. 8B) was isolated and cultured on MS medium and containing either 0.1 mg/l NAA and BAP each or 0.5 mg/l NAA and 1.0 mg/l BAP. In three weeks compact callus changed to sponge like mass. The callus showed somewhat better growth and some green areas appeared in cultures on medium with 0.5 mg/l NAA and 1.0 mg/l BAP. After 5 weeks the callus growth was noted to be less in either treatment as compared with the similar cultures growing on RC medium (Figs. 7B-7D). However the callus continued the growth for another 11 weeks.

d) Effect of auxins in combination with cytokinins in the presence of either CH, CM or YE.

The stem explants were cultured on medium containing 1000 mg/l CH and 1.0 mg/l NAA with 0.5 mg/l Kn. Some callus was produced after 13 days (Fig. 5A). This callus which continued slow growth for 5 weeks was then sub-cultured on standard AM medium as well as on AM medium with 2.0 mg/l NAA. Both media sustained better callus growth which apparently was less with increased NAA concentration (Figs. 6C and 6D).

The callus pieces were later sub-cultured on MS medium containing CM and either 1.0 mg/l 2,4-D or 1.0 mg/l NAA and 0.5 mg/l Kn. The callus on medium with 2,4-D grew for 19 weeks. The growth rate was comparatively slower than for same calli cultured with NAA and Kn (Figs. 6A and 6B). The callus from cultures treated with 2,4-D was sub-cultured in MS medium with 0.5 mg/l Kn and 2000 mg/l YE. This treatment did not improve the callus growth (Fig. 7A).

The callus sub-cultured with NAA and Kn exhibited active growth for 8 weeks and then the growth rate slowed down.

The NAA and Kn callus was sub-cultured on medium containing either 0.5 mg/l NAA and 1.0 mg/l BAP or 0.1 mg/l NAA and BAP each. CM was also added to the medium. On medium with 0.5 mg/l of NAA and 1.0 mg/l of BAP, the callus grew for 10 weeks and then started browning. The callus was

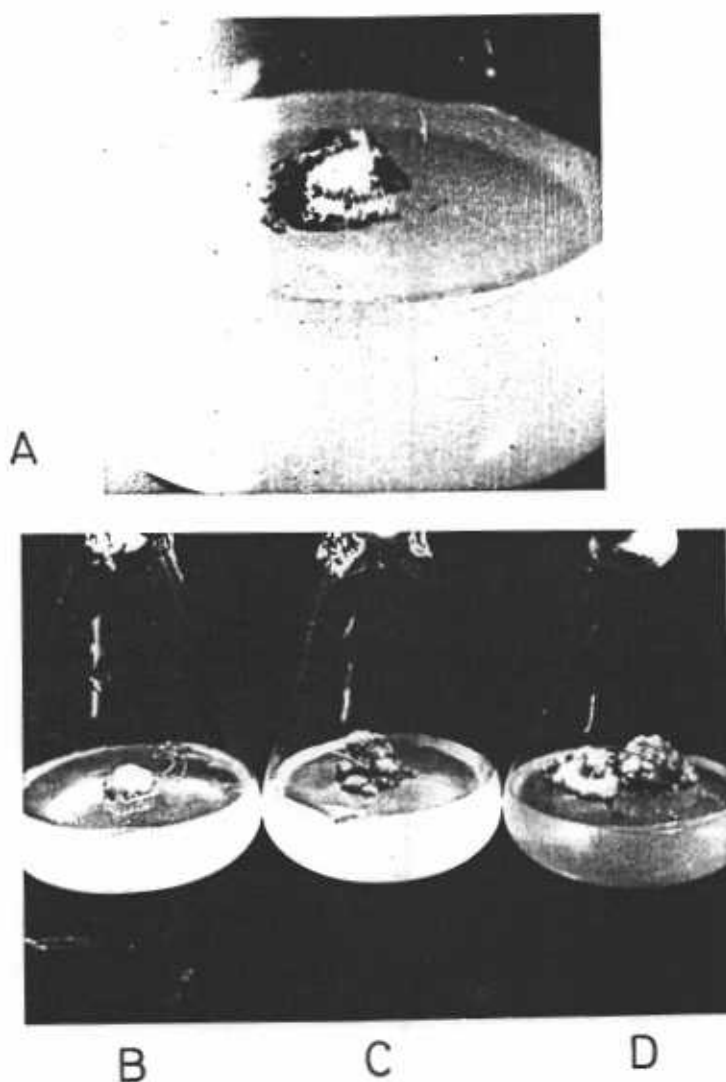


Fig. 7. Stem callus growing on MS medium supplemented with :
 (A) 1.0 mg/l 2,4-D, 0.5 mg/l Kn and 2000 mg/l YE
 (B) 0.5 mg/l NAA and 1 mg/l BAP
 (C) 0.1 mg/l each of NAA and BAP
 (D) On White's Root Culture medium with same regulators concert as in (C).

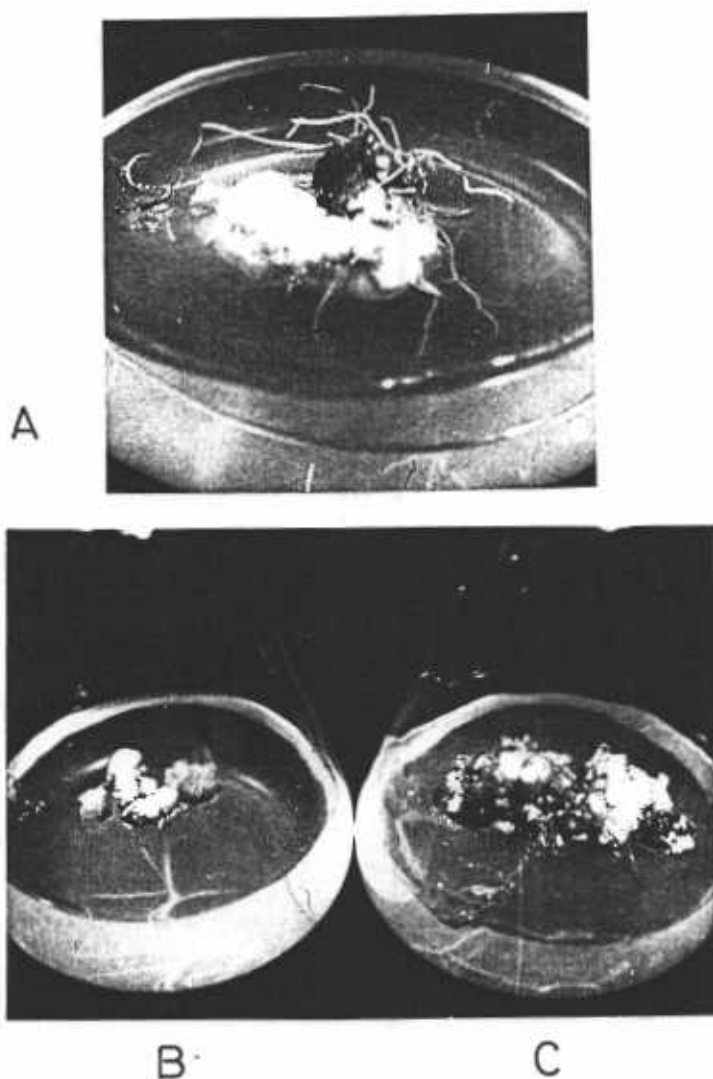


Fig. 8. Stem callus growing on White's Root Culture medium containing 0.1 mg/l NAA and BAP and:
 (A) and (B) without CM.
 (C) with 100 ml/l CM.

sub-cultured on medium containing SDDC. The callus restarted growth which continued for 36 weeks (Table-4). This callus from NAA and BAP treated flasks was then cultured on AM medium. It not only started growing but the growth was quite active for 15 weeks followed by a slow growth period with ultimate cessation of growth after 32 weeks.

In callus cultured with 0.1 mg/l each of NAA and BAP no growth could be observed and callus ultimately turned black.

Effect of Change in Medium on Stem Explants

a) Response to NAA alone or in combination with BAP, in presence of CM and biotin.

The slow growing and browning stem pieces taken from cultures growing on MS medium containing CM, 1.0 mg/l NAA and 0.5 mg/l Kn were subcultured on RC medium containing SDDC and 0.8 mg/l NAA. The further browning of callus stopped after 10 days (Figs. 9A and 9B). The callus commenced growth (Fig. 9C), which was quite active for 13 weeks and then slowed down. The callus was still showing some growth after 61 weeks.

The callus pieces taken from cultures growing on MS medium containing 0.1 mg/l NAA and BAP were subcultured on RC medium containing either similar concentration of regulators or with 0.8 mg/l of NAA only. The RC medium contained

Table 4: Growth in stem callus of *Rawolfia serpentina* on MS, White's Root Culture (RC) and Abou-Mandour (AM) media with different treatments of growth regulators, 100 ml/l CM, 500 mg/l CH, 2000 mg/l YE, 10 mg/l biotin and 250 mg/l Sodium diethyldithiocarbamate (SDDE).

Treatment			Callus	
Medium	Growth regulators (mg/l)	Additives	Growth	Growth period (weeks)
MS	2,4-D 0.5	-	+	8
	1.0	-	+	9
	1.0	CM	+	19
	1.0	CH	+	4
	2,4-D+Kn 1.0+0.5	YE	-	-
	1.0+1.0	CH	+	4
NAA	1.0	-	+	11
NAA+Kn	1.0+0.5	CH*	+	5
	1.0+0.5	CM	++	8
	0.5+0.5	-	+	8
	0.5+1.0	-	+	8
	0.5+0.5	CM	+	4
	0.5+1.0	CM	+	4

Table 4: continued

Treatment			Callus		Growth period (weeks)
Medium	Growth regulators (mg/l)	Additives	Growth		
MS	NAA+BAP	0.1+0.1	CM	+	8
		0.1+0.5	-	+	5
		0.1+1.0	-	+	5
		0.5+0.1	-	++	5
		0.5+0.5	-	++	5
		0.5+1.0	-	++	10
		0.5+1.0	CM	+	10
		0.5+1.0	CM+SDDC	++	36
		0.5+1.5	-	+	10
		0.5+2.0	-	+	10
		1.0+0.1	-	+	5
		1.0+0.5	-	+	5
RC		1.0+1.0	-	+	5
	NAA	0.8	CM+biotin	+	7
		0.8	CM+biotin+SDDC	+++	13

Table 4: continued

Treatment			Callus	
Medium	Growth regulators (mg/l)	Additives	Growth	Growth period (weeks)
AM	NAA+BAP 0.1+0.1	-	+++	24
	0.1+0.1	CM	+++	36
	0.1+0.1	CM+biotin	++	17
	Standard/	CH*	+++	15
	Standard//	CH*	++	12

Legend:-

- no growth

+ fair growth

++ good growth

+++ copious growth

* concentration 1000 mg/l

/ 1 NAA+0.1 EAP+0.3Kn+4 AS+6 2,4-D (mg/l)

// 2 mg/l NAA+other regulators as in/.

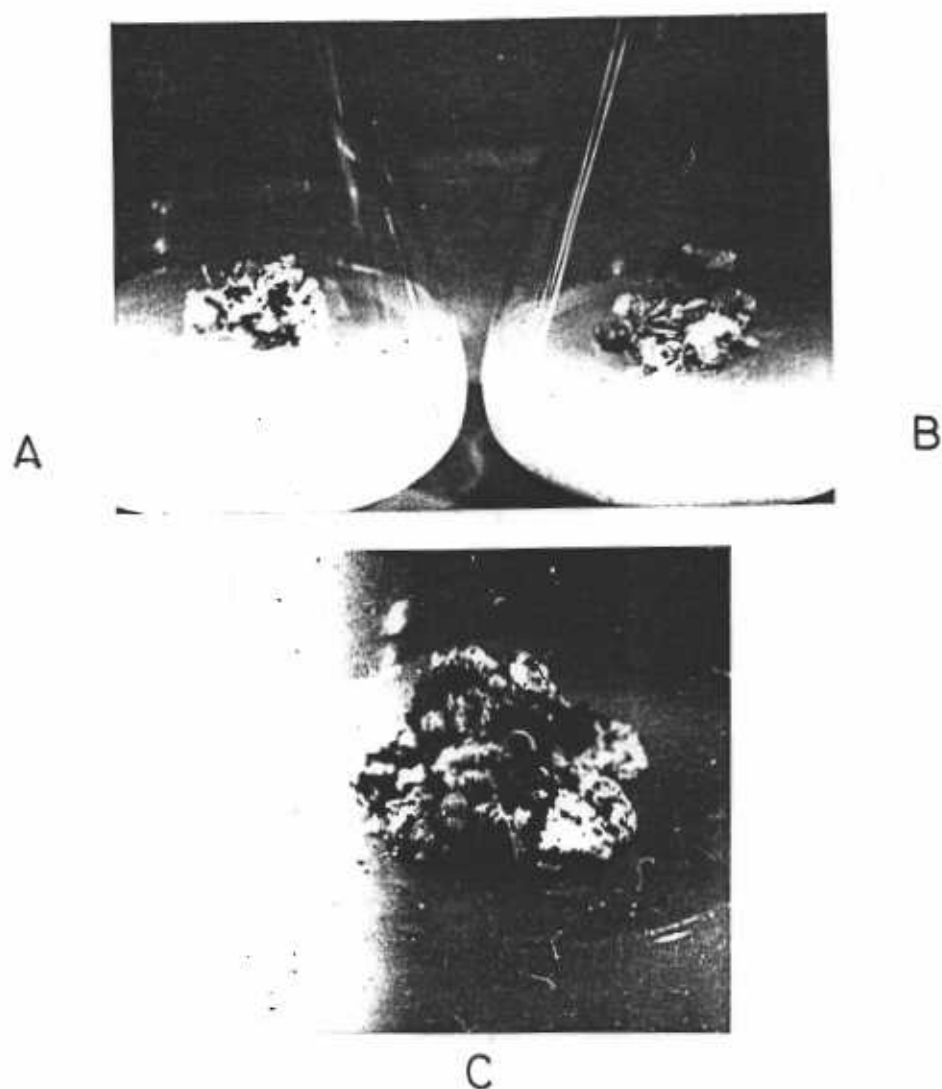


Fig. 9. Stem callus showing revival of growth within 10 days on White's Root Culture medium supplemented with 0.8 mg/l NAA, 10 mg/l biotin, 100 ml/l CM and:
 (A) 250 mg/l Sod. diethyldithiocarbamate (SDDC)
 (B) without SDDC.
 (C) the growth with SDDC in 3 weeks. New callus growth is seen as white areas.

biotin and CM. In callus cultured with both NAA and BAP, new white callus was formed after 5 weeks. Callus then developed green pigmentation and continued its growth for another 12 weeks.

In callus subcultured with NAA only, the callus started growth after 5 weeks. The growth was negligible upto 7 weeks.

1-3. Formation of Callus by Root Explants and its further growth.

The root explants used in the present studies were taken from actively field growing plants in summer. Roots excised from aseptically raised seedlings in the laboratory were also cultured. The RC, AM or MS media were used for the culture. Concentrations of the additives used were: CM 100 ml/l, CH 1000 mg/l, YE 2000 mg/l, SDDC 250 mg/l unless otherwise mentioned. The pH of media was adjusted to 5.8.

(1) Culture of Root Explants from Field Plants:

a) Effect of auxins in the presence of CM in MS medium.

Root explants were cultured on MS medium containing 1.0 mg/l of either 2,4-D or NAA. The cultures were observed upto 13 weeks. Regular subculturing on fresh medium was done after every 4 weeks. No callus was formed on these cultures during the observation period.

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b) Effect of auxins in the presence of CH.

The root explants were cultured on RC medium containing 0.01, 0.05, 0.1, 0.5 or 1.0 mg/l of either 2,4-D or NAA. After culturing, these tubes were kept in the dark for 10 days. Since no callusing or swelling of explants occurred during this period, these cultures were placed in 16 hours light.

Under light the cultures with 1.0 mg/l 2,4-D, formed some callus within one week. As this callus showed little growth in next 3 weeks, it was then cultured with 500 mg/l CH on MS medium containing 1.0 mg/l 2,4-D. Although no further growth of already formed callus occurred within next 10 weeks but new mass of callus was observed on this medium after 3 weeks (Fig. 10A). The explants represented CH and 1.0 mg/l of 2,4-D. During 8 weeks observation, no callus growth or fresh callus formation was observed on these explants. They were, therefore, transferred to AM medium. Still callus growth was not effected.

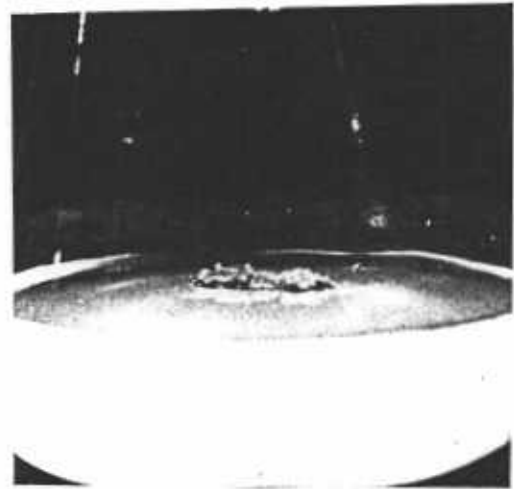
In root explants cultured with NAA, callus was observed in 1.0 mg/l concentration within 2 weeks after transfer to light.

c) Effect of NAA in combination with Kn in the absence or presence of CH.

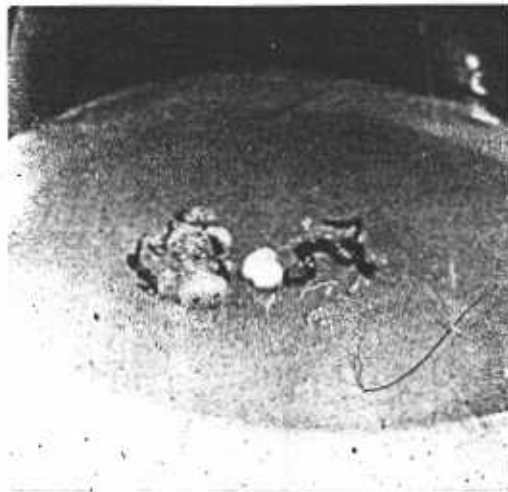
Root explants were cultured with 0.01, 0.05, 0.1, 0.5 or 1.0 mg/l NAA each combined with the identical concen-



A



B



C

Fig. 10. Callus formation in 3 weeks on root explants taken from field growing plants of *Rauwolfia serpentina* cultured on MS medium containing 500 mg/l CH and:

(A) 1.0 mg/l 2,4-D

(B) 1.0 mg/l NAA and 0.05 mg/l Kn;

(C) Explants from aseptic seedlings cultured on White's Root Culture medium containing 100 ml/l CM and 1.0 mg/l 2,4-D.

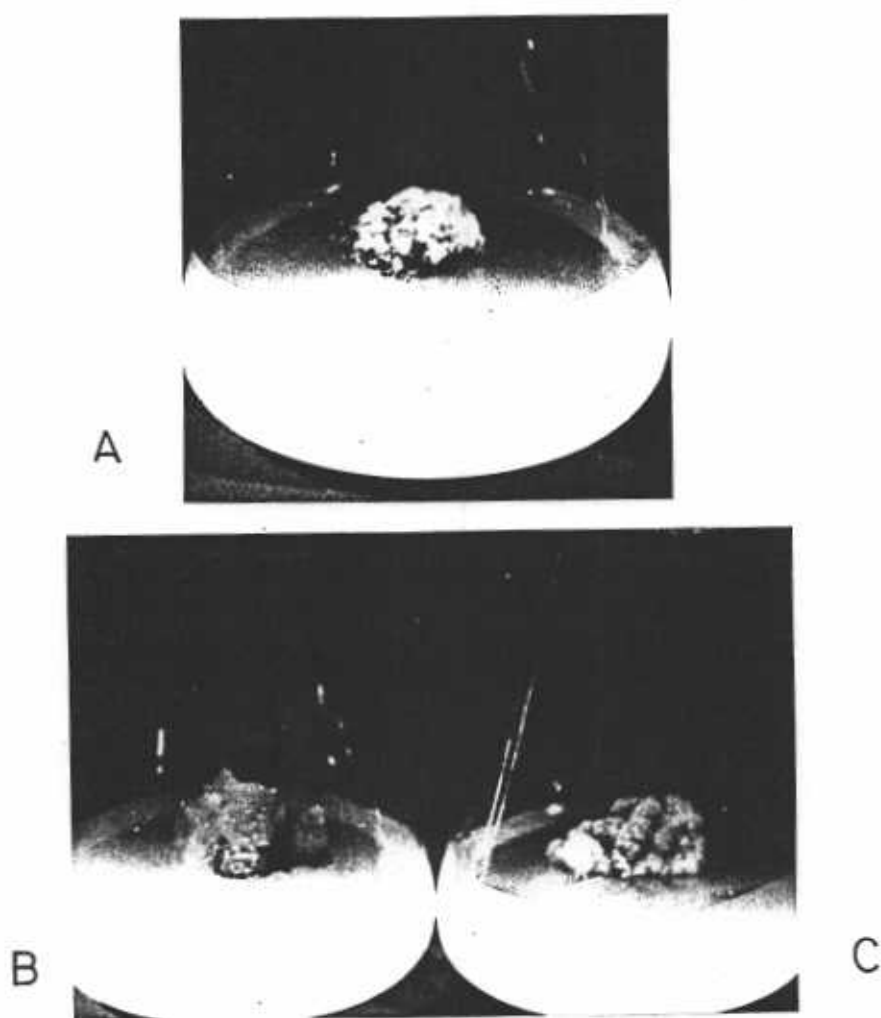


Fig. 11. Root callus showing growth on:

- (A) MS medium supplemented with 2000 mg/l YE, 1.0 mg/l 2,4-D and 0.5 mg/Kn and;
- (B) On Abou-Mandour medium with standard regulators and supplemented with 1000 mg/l CH

Callus grown in dark is hyaline (B) and root callus grown in standard light condition is yellowish-white (C).

tration of Kn. The cultures, when kept in dark for 10 days, did not form callus. They were then transferred to 16 hours light. After growing for about a week under light, explants initiated callus in cultures containing 0.1, 1.0 mg/l NAA with 0.01 mg/l Kn and 1.0 mg/l NAA with 0.05 mg/l Kn.

During the third week, cultures treated with the following concentrations initiated callus formation: 0.05, 0.1 mg/l NAA and 0.05 mg/l Kn; 0.1, 1.0 mg/l NAA and 0.1 mg/l Kn; and 0.5 mg/l NAA and Kn.

In the following 3 weeks, the callus grew better with these three treatments: 1.0 mg/l NAA and 0.01 mg/l Kn; 0.5 and 1.0 mg/l NAA each with 0.05 mg/l Kn. Callus pieces taken from these treatment were cultured on MS medium containing 500 mg/l CH. The regulators added were either 1.0 mg/l NAA and 0.01 mg/l Kn or 0.5, 1.0 mg/l NAA and 0.05 mg/l Kn.

Within 10 weeks callus growth was not observed in these transfers (Fig. 11B) and the callus then was recultured on RC medium containing CH, but without any success. Likewise when transferred to AM medium no callus growth occurred.

d) Effect of NAA in combination with BAP and CM.

Explants cultured on media containing 0.1 mg/l NAA and BAP with CM did not produce any callus within 13 weeks observation period.

(2) Culture of Root Explants from Aseptic Seedlings.

a) Effect of auxins and/or kinetin in the presence of CM.

Addition of either 1.0 mg/l 2,4-D or 0.5 and 1.0 mg/l NAA with 0.05 mg/l Kn to the medium did not cause substantial callus formation in explants taken from 140 days old seedlings.

b) Effect of auxins and/or kinetin in the presence of CH or CM.

Root explants were taken from 210 days old seedlings and cultured on medium containing either 1.0 mg/l 2,4-D or 1.0 mg/l NAA and 0.05 mg/l Kn.

The explants cultured with 2,4-D did not show any growth during the 4 weeks while those cultured with NAA and Kn. showed slight callus formation within 9 weeks. The replacement of CH with CM had no effect on further growth of this callus.

c) Effect of 2,4-D in the presence of CM.

The root explants taken from 275 days old seedling were cultured on medium containing 1.0 mg/l 2,4-D. These explants initiated callus after 2 weeks (Table-5). The callus continued its growth (Fig. 10C) for 14 weeks and appeared as white compact mass.

able 5: Callus formation on root explants of *Rauwolfia serpentina* on White's Root Culture (RC) medium with different treatments of growth regulators, 100 ml/1 CM and 1000 mg/1 CH.

Explant's origin	Treatment		Callus		Remarks
	Growth regulators (mg/l)	Nutrient	Formation	Initiation period (days)	
Field Plants	2,4-D 0.1	CH	-	-	
	0.5	CH	-	-	
	1.0	CH	+	7	
	NAA	0.1	-	-	
		0.5	-	-	
		1.0	-	-	
	NAA+Kn	0.01+0.01	-	-	
		0.05+0.05	+	21	
		0.1+0.01	+	7	
		0.1+0.05	+	21	
	0.1+0.1	-	+	21	
	0.1+0.5	-	-	-	
	0.1+1.0	-	-	-	
	0.5+0.5	-	+	21	

Table 5: continued.

Explant's origin	Treatment		Callus		Remarks
	Growth regulators (mg/l)	Nutrient	Formation	Initiation period (days)	
Aseptically grown seedlings	1.0+0.01	-	+	7	
	1.0+0.05	-	+	7	
	1.0+0.1	-	+	21	
	1.0+0.5	-	-	-	
	1.0+1.0	-	-	-	
	0.5+0.05	CM	-	-	Seedling age in days =140
2,4-D	1.0+0.05	CM	-	-	=140
	1.0+0.05	CH	+	63	=210
	1.0	CH	-	-	=210
	1.0	CM	-	-	=140
	1.0	CM	++	14	=275
<i>In Vitro</i> *	1.0	CM	+++	21	
	NAA+BAP 0.1+0.1	-	++	28	
	0.1+0.1	CM	++	28	

Table 5: continued

Explant's origin	Treatment		Callus		Remarks
	Growth regulators (mg/l)	Nutrient	Formation	Initiation period (days)	
	0.01+0.01	-	+	28	
	(+0.1GA ₃)				

Legend:-

- no callus.
- + fair callus.
- ++ good callus.
- +++ copious callus.
- * Roots differentiated in stem callus.

d) Callus initiation in explants on AM medium in dark and light.

Explants were taken from 250-day-old seedlings and cultured on AM medium showed no callus formation upto 8 weeks.

When the explants, taken from 275 days old seedlings: in which the callus was already induced by the addition of 1.0 mg/l 2,4-D on RC medium containing CM, were transferred to AM medium, showed active callus growth upto 40 weeks (Table-6). However, the growth thereafter slowed down. In the begining, the callus was friable and granular and of a yellowish-white colour (Fig. 11C).

A batch of root callus cultured on AM medium was kept in dark. Callus initiated growth on 3rd day after subculturing. In the following 2 weeks, callus appeared as a hyaline mass (Fig. 11B). The callus kept growing, although slowly, till 50th week (Fig. 12).

e) Effect of 2,4-D and/or Kn with addition of YE and SDDC in MS medium.

The AM callus cultures growing under light conditions were transferred to MS medium. The medium contained YE and 1.0 mg/l 2,4-D and 0.5 mg/l Kn. After one week of transfer, slow growth in callus was observed with slight browning. In the 3rd week browning disappeared (Fig. 11A) and callus growth

FAIR +
 GOOD ++
 COPIOUS +++

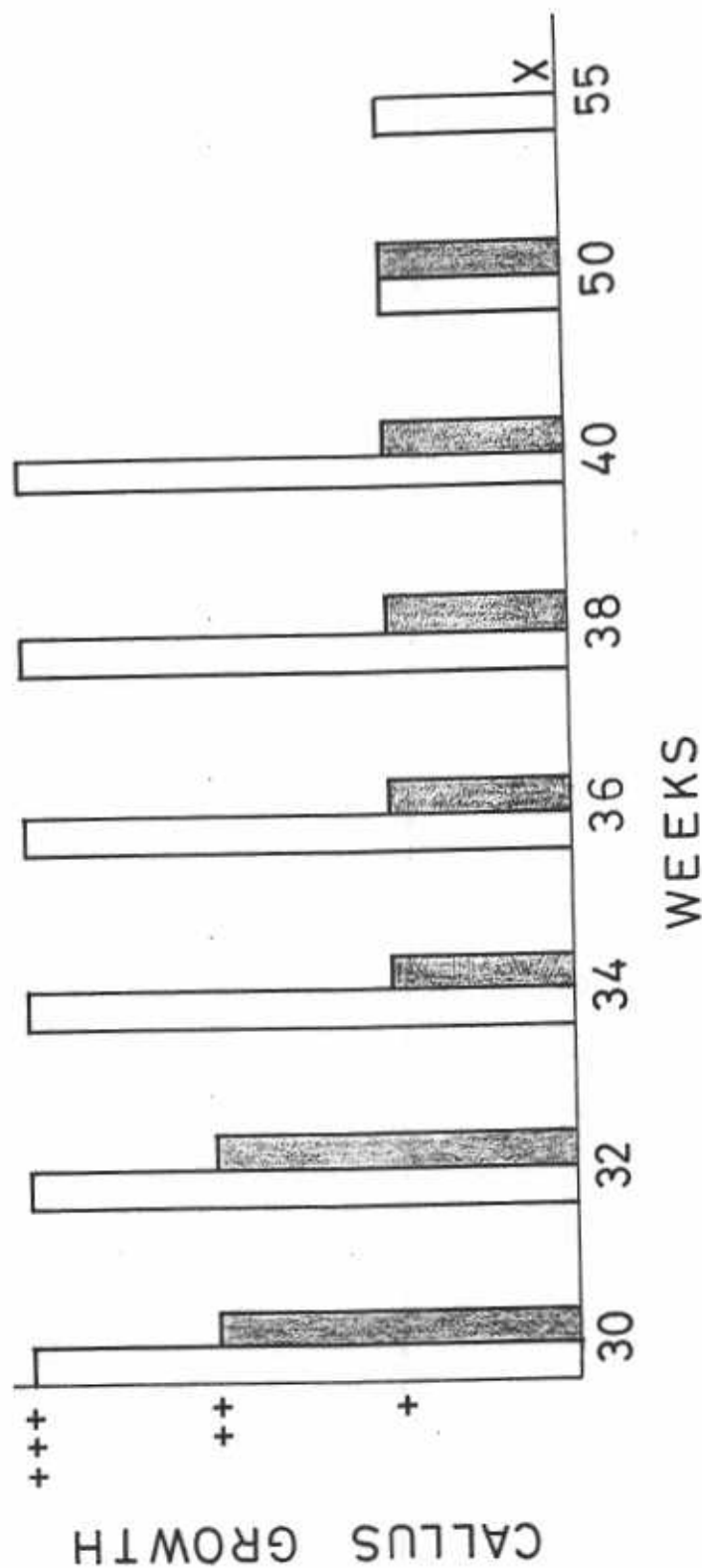


Fig. 12. Growth in root callus of *Rauwolfia serpentina* on Abou-Mandour's medium supplemented with 1 NAA, 0.3 Kn, 0.1 BAP; 4 AS, 6 2,4-D and 1000 mg/l CH, in 16 hrs. light cycled (Blank) and 24 hrs. dark (Solid) conditions.

Table 6: Growth in root callus of *Rawolfia serpentina* on White's Root Culture (RC), MS and Abou-Mandour (AM) media with different treatments of growth regulators, 100 ml/1 CM, 1000 mg/1 CH, 2000 mg/1 YE and 250 mg/1 SDDC.

Callus origin	Treatment			Callus		
	Medium	Growth regulators (mg/l)	Additives	Growth	Growth period (weeks)	
Field Plants	RC	2,4-D	1.0	CH	+	3
		NAA+Kn	0.05+0.05	-	+	3
			0.5+0.05	CH	-	-
			0.5+0.05	-	++	3
			1.0+0.01	CH	-	-
			1.0+0.01	-	++	3
			1.0+0.05	CH	-	-
			1.0+0.05	-	++	3
			1.0+0.05	CM	-	-
			1.0+0.1	-	+	3
			1.0+0.05	CH	-	-
			0.5+0.05	CH	-	-
MS		2,4-D	1.0	CH	-	-

Table 6: continued.

Callus origin	Treatment		Callus	
	Medium	Growth regulators (mg/l)	Additives	Growth period (weeks)
Aseptic seedlings	AN	2,4-D+Kn 1.0+0.5	YE	+
		1.0+0.5	YE+SDDC	+
		Standard*	CH	-
		Standard*	CH	+++
In Vitro**	RC	2,4-D 1.0	CM	++
		NAA+BAP 0.1+0.1	CM	+++
		0.1+0.1	-	++
		0.01+0.01	-	++
		(+0.1 GA ₃)		

Legend:-

- no growth.

+ fair growth.

++ good growth.

+++ copious growth.

* 1 NAA+0.1 BAP+0.3 Kn+4 AS+6 2,4-D (mg/l).

** Callus obtained from *in vitro* differentiated roots.

continued upto 7th week. Addition of SDDC in the following subcultures did not improve the callus growth, which continued at a slow pace for 46 weeks.

II. ROOT DIFFERENTIATION IN CALLUS CULTURES

Differentiation of roots was observed in the calli formed by explants of stem, leaf and root of *R. serpentina*. The stem callus was found to be more conducive to root differentiation; root callus only sporadically exhibited root differentiation and leaf callus showed no root differentiation.

The medium employed for the differentiation studies was MS unless otherwise mentioned. CM, when used was 100 ml/l of medium. Biotin was used as 10.0 mg/l. pH of the media was adjusted to 5.8.

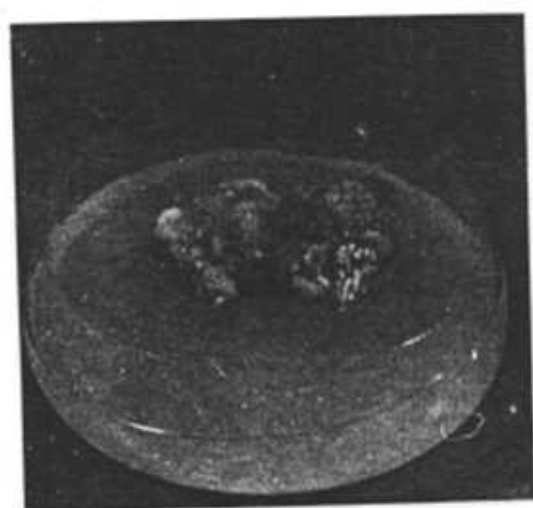
II-1. Root Differentiation in Stem Callus Cultures.

a) Effect of 2,4-D and AM medium

Root differentiation was not noticed in a stem calli cultured on MS medium containing CM and 1.0 mg/l 2,4-D (Fig. 13D) or on AM medium during observation period of 22 and 32 weeks respectively.

b) Effect of NAA with Kn or BAP in the absence or presence of CM.

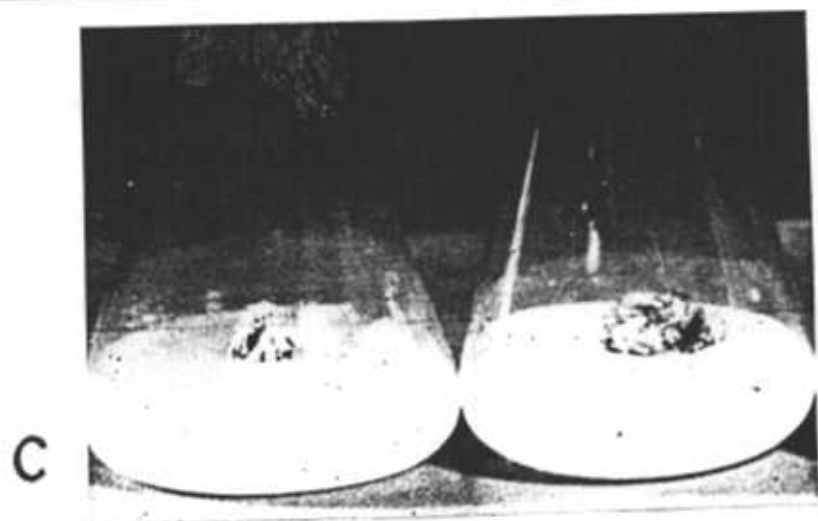
The stem callus cultured on medium containing CM, 1.0 mg/l NAA and 0.5 mg/l Kn, differentiated roots after



A



B



C

D

Fig. 13. Root differentiation in stem callus cultured on MS medium supplemented with 100 ml/l CM and:

(A) 1.0 mg/l NAA and 0.5 mg/l Kn

(B) 0.5 mg/l NAA and 1.0 mg/l BAP

(C) 0.1 mg/l of NAA and BAP

(D) 1.0 mg/l 2,4-D; Note the absence of root differentiation in (D).

about 8 weeks (Fig. 13A). The differentiation continued for further 27 weeks during callus growth, but only sporadically in the last 4 weeks.

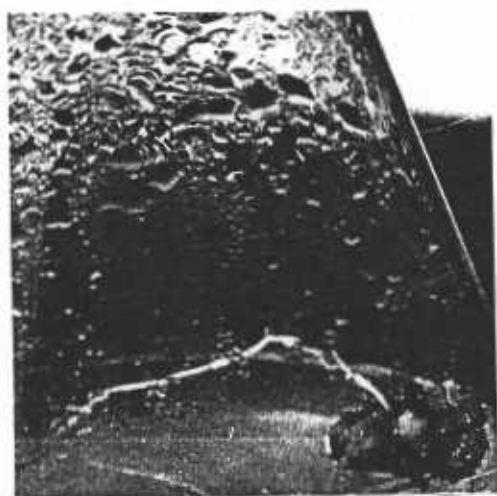
The stem callus cultured on a medium containing CM, 0.5 mg/l NAA and 1.0 mg/l BAP showed differentiation of roots after 7 weeks of callus culture (Fig. 13B). The differentiation continued progressively for the next one week and later intermittantly for about 28 weeks.

The stem callus cultured on medium containing CM and 0.1 mg/l NAA and BAP also showed differentiation of roots beginning after 12 days until 6 weeks (Fig. 13C). Callus cultured on medium containing 0.1 mg/l NAA and BAP, without CM, did not exhibit root differentiation for 35 weeks.

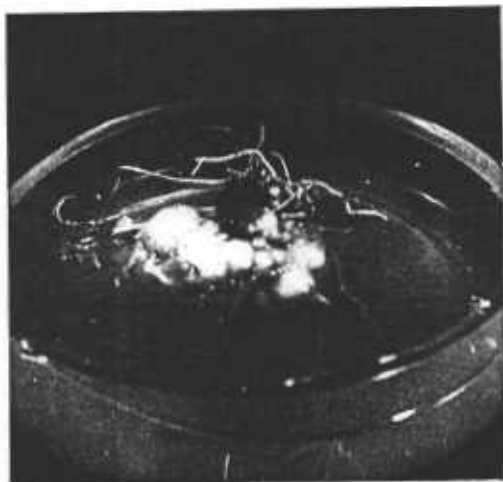
c) Effect of NAA with BAP in the absence or presence of CM in the RC medium.

The stem callus when cultured on medium containing 0.1 mg/l NAA and BAP started root differentiation after 4 weeks which continued for another 4 weeks (Fig. 14A). While differentiating roots, the callus itself continued growth and in 4 more weeks, roots started differentiating within the newly formed milky-white callus (Fig. 14B).

The stem callus growing with 0.1 mg/l NAA and BAP, when subcultured either with or without CM, showed root



A



B



C



D

Fig. 14. Root differentiation in stem callus cultured on White's Root Culture medium containing 0.1 mg/l NAA and BAP:

(A) Unpigmented

(B) Milky-white

(C) Green callus

(D) Green callus in cultures containing 100 ml/l CM.

differentiation after 2 weeks in both the treatments. The cultures were continuously differentiating roots in the following week. Thereafter, root differentiating capacity was found diminishing gradually with the change in the appearance of callus from compact to loose mass in CM cultures and with development of pigments in flasks without CM.

Root differentiation restarted later in CM cultures and continued for approximately 10 weeks. The calli with different pigments were separated and each was cultured separately with or without CM. Root differentiation in green callus was observed after two weeks in presence as well as absence of CM and in pinkish-green and hyaline callus with greenish tinge only on the medium without CM (Fig. 14C and 14D). Root differentiation continued for 8 more weeks in green callus both with and without CM and in pinkish-green and hyaline with greenish tinge, growing on a medium without CM. In white callus the root differentiation was observed after 5 weeks of culture on the medium containing no CM and afterwards, the root differentiation continued in this callus for 5 more weeks.

- d) Effect of adding NAA alone or with BAP to RC medium containing CM and biotin.

The stem callus when cultured on a medium containing

0.8 mg/l NAA showed root differentiation after 1 week (Fig.15A and 15C) which continued for about 84 weeks (Fig.16).

The rooting capacity of stem-callus apparently increased with addition of biotin and 0.8 mg/l NAA to RC medium in contrast to the stem callus cultured on MS medium containing 1.0 mg/l NAA and 0.5 mg/l Kn. Because the latter callus differentiated roots for a lesser period (Table-7).

When cultured without biotin on RC medium containing 0.1 mg/l NAA and 0.1 mg/l BAP, the stem callus formed roots after 1 week (Fig. 15B). The differentiation continued for 59 more weeks. The rooting capacity of stem callus with 0.1 mg/l NAA and BAP was also less than with 0.8 mg/l NAA (Figs. 15A and 15B).

II-2. Root Differentiation in Root Callus Cultures.

a) Effect of 2,4-D in the presence of CM in RC medium.

The root callus cultured on medium containing 1.0 mg/l 2,4-D showed root differentiation in 11 weeks. The differentiation thereafter continued for 10 more weeks.

b) Effect of AM medium.

The root callus was then cultured on AM medium. One batch of cultures was kept in dark. Root differentiation was not noticed in either light or dark cultures upto 52 week.

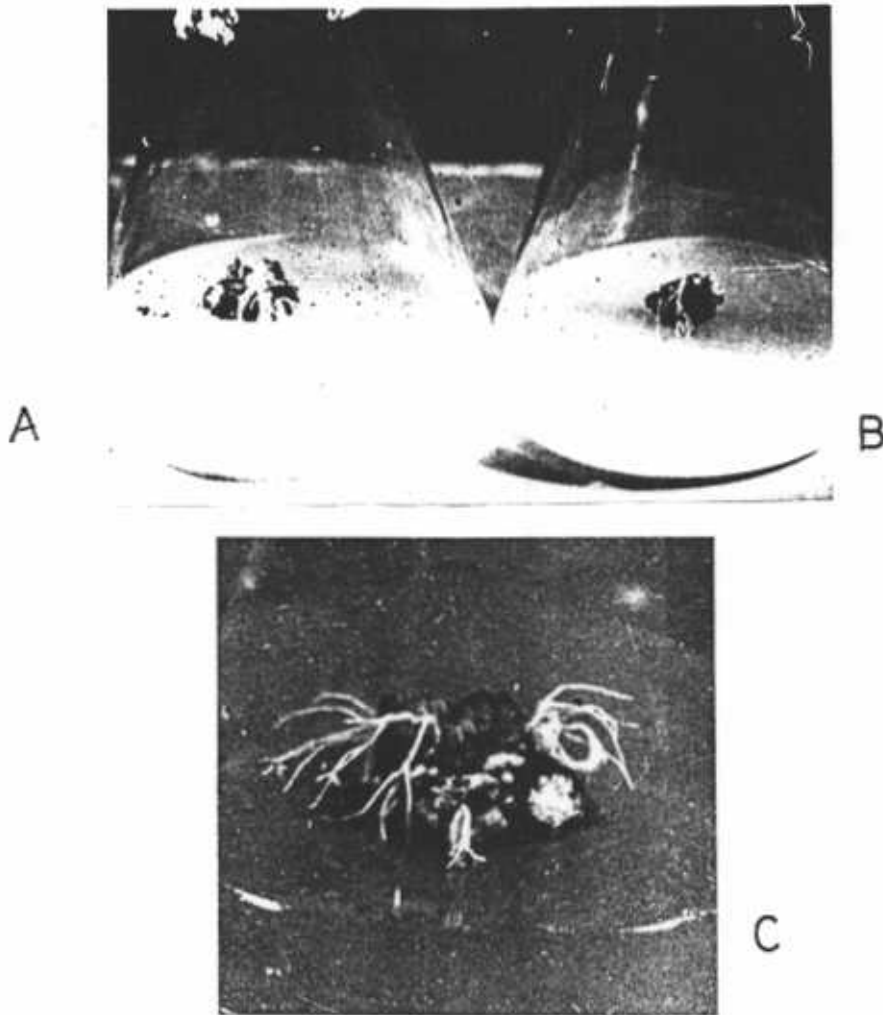


Fig. 15. Stem callus showing root differentiation in one week on White's Root Culture medium, supplemented with 100 ml/l CM, 10.0 mg/l biotin and:

- (A) 0.8 mg/l NAA
- (B) 0.1 mg/l NAA and BAP
- (C) Cultures with treatment as in (A) after 3 weeks.

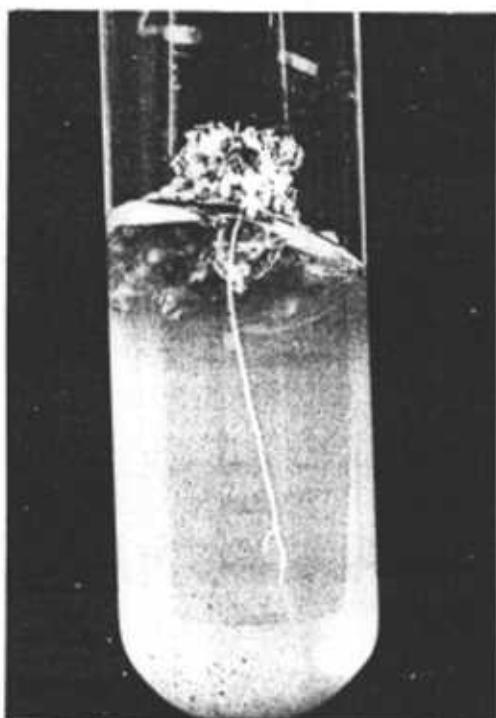


Fig. 16. Stem callus subcultured for 49 weeks on White's Root Culture medium supplemented with 100 ml/l CM, 10 mg/l biotin and 0.8 mg/l NAA. New roots were still forming when this picture was taken.

Table 7: Root differentiation in stem callus of *Rauwolfia serpentina* on White's Root Culture (RC) and MS media with different treatments of growth regulators, 100 ml/l CM and 10 mg/l biotin.

Treatment			Root differentiation	
Medium	Growth regulators	Nutrient	Initiation (weeks)	Continuity (weeks)
RC	NAA 0.8	CM+biotin	1	84
	NAA+BAP 0.1+0.1	-	2	9
	0.1+0.1	CM	2	11
MS	0.1+0.1	CM+biotin	1	59
	0.1+0.1	-	-	-
	0.1+0.1	CM	1.5	6
	0.5+1.0	-	7	29
	NAA+Kn 1.0+0.5	CM	8	27

III. ROOT CULTURE

The explants taken from *in vitro* differentiated roots in the stem callus cultures of *R. serpentina* were studied on RC medium. In some tubes CM 100 ml and biotin as 10 mg/l were added. The pH of the medium was 4.9.

A) Effect of NAA and/or BAP in the absence or presence of CM.

a) Explants from stem callus roots.

These root explants were cultured in liquid medium containing 0.1 mg/l NAA and BAP. The root initiated laterals in 4 weeks (Fig. 17A).

When roots were cultured on medium containing 0.1, 0.5 or 0.8 mg/l NAA alone or in combination with either 0.01 or 0.05 or 0.1 mg/l BAP, the apical growth in roots was observed after 6 days in treatments having 0.1 or 0.5 mg/l NAA; 0.5 mg/l NAA and 0.01 mg/l BAP; 0.8 mg/l NAA and 0.05 mg/l BAP and by 2 weeks in cultures growing on 0.1 mg/l NAA and BAP. Root laterals were noticed after about 6 days in cultures having 0.8 mg/l NAA and 0.1 mg/l BAP and by 2nd week in treatments having 0.1 or 0.5 mg/l NAA; 0.01 mg/l BAP and 0.1 or 0.8 mg/l NAA and 0.1 mg/l NAA and BAP each.

Lateral root formation was better and continuously



A



B



C

Fig. 17. Root segments in culture, producing laterals on White's Root Culture medium supplemented with:

- (A) 0.1 mg/l NAA and 0.1 mg/l BAP
- (B) 0.5 mg/l NAA
- (C) 0.8 mg/l NAA and 10.0 mg/l biotin.

increased upto 9 weeks in cultures growing with 0.5 mg/l NAA (Fig. 17B).

b) Root Explants with attached stem callus.

i) With white callus

Explants cultured on media containing after CM alone or in combination with 0.1 mg/l NAA and BAP, each showed apical growth as well as formation of laterals within 5 weeks. Number of laterals increased during the following weeks and this average increase was about 110.86% (Table-3). Exclusion of CM from the medium gave only 15.83% laterals.

ii) With green callus

The root explants produced laterals on the medium containing CM and 0.1 mg/l NAA and BAP in 2 weeks. The number increased in the following two weeks by an average of 42.65%. Exclusion of CM delayed the lateral formation which appeared after 8 weeks.

B) Effect of NAA in the absence or presence of biotin and CM.

a) On apical root segments.

The apical root segments cultured on the medium containing 0.1, 0.5 or 0.8 mg/l NAA, showed apical growth in the

Table 8: Lateral roots formation on *in vitro* differentiated roots from stem callus of *Rauwolfia serpentina* on RC medium supplemented with 0.1 mg/l NAA, 0.1 mg/l BAP and with (+) or without (-) 100 ml/l coconut milk (CM). Column three under lateral roots, represents increase in number of roots formed in one week.

CM Main Roots(Weeks)		Lateral Roots. (weeks)			Average \$ increase	
5	5	6	Increase in number	\$ Increase		
+		7	10	3	42.85	110.86
		12	18	6	50.0	
		6	16	10	166.66	
		2	2	-	-	
		3	3	-	-	
	11	-	-	-	-	
		-	-	-	-	
		-	-	-	-	
		5	23	18	360.0	
		1	7	6	600.0	
		1	1	-	-	

Table 8: continued.

CM	Main Roots (weeks)		Lateral Roots (weeks)		Increase in number	% Increase	Average % increase
	5	6	5	6			
-			8	9	1	12.5	
			4	4	-	-	
			4	4	-	-	15.83
			4	4	-	-	
			3	5	2	66.66	

3rd week with 0.8 and in 7th week with 0.5 mg/l NAA.

Laterals formation occurred in 6 days with 0.8 and in 7 weeks with 0.5 mg/l NAA. No lateral roots were formed with 0.1 mg/l NAA. Laterals formation continued upto 13th week with 0.5 and 23rd week with 0.8 mg/l NAA (Fig. 18). The laterals formed in 0.8 mg/l NAA cultures, exhibited apical growth and in turn produced secondary lateral roots.

b) Sub-apical root segments.

Root segments when cultured on a medium containing 0.5 or 0.8 mg/l NAA produced laterals in one week which continued for another 5 weeks. Roots, when cultured on the medium containing CM, biotin and 0.8 mg/l, NAA produced laterals in 5 weeks, which continued for the following 8 weeks. Suspension medium containing biotin and 0.8 mg/l NAA, induced lateral formation after about 5 weeks in sub-apical root segments (Fig. 17C).

INITIATION OF 1st. ROOT —
 CONTINUATION OF ROOT FORMATION - - -
 APICAL ROOT SEGMENTS Δ
 SUBAPICAL ROOT SEGMENTS ○

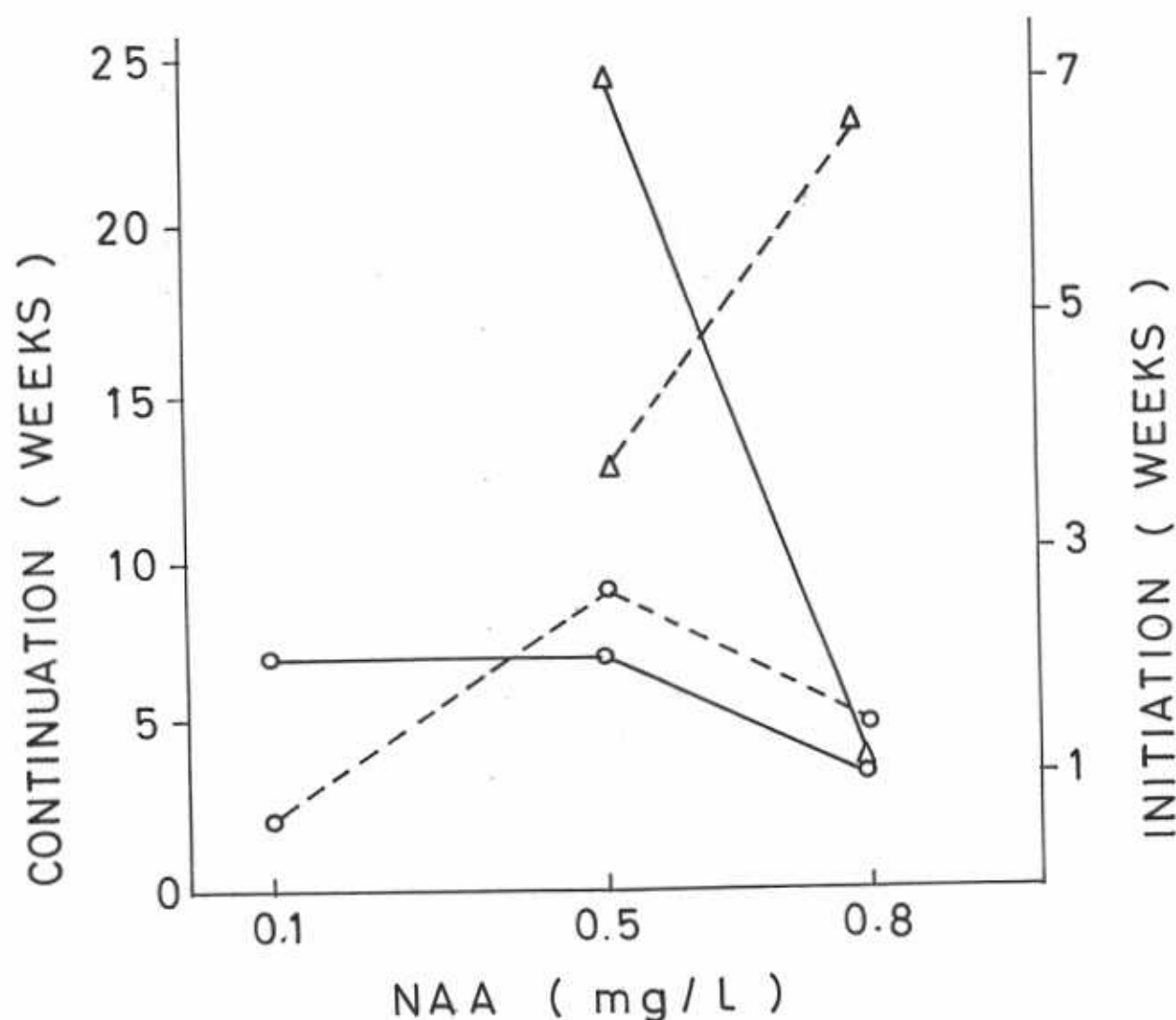


Fig. 18. Lateral root formation *in vitro* differentiated roots in stem callus of *Rauwolfia serpentina* on White's Root Culture medium supplemented with 100 ml/l coconut milk and 10 mg/l biotin.

IV. CALLUS FORMATION ON *IN VITRO* DIFFERENTIATED ROOTS.

The root explants for the present studies were taken from *in vitro* differentiated roots in stem callus. RC medium with or without CM 100 ml/l and CH 1000 mg/l was used. The pH of the media was adjusted at 5.6. The root explants were cultured on a medium containing either 0.1 mg/l NAA and BAP each, or 1.0 mg/l 2,4-D alone.

IV.1. NAA and BAP Induced Root Callus.

a) White callus

The root explants during the present treatment were cultured in a liquid medium. This medium did not contain CM. The callus initiation was observed after about 4 weeks. Similarly when the root explants were cultured on a solidified medium, with or without CM again the callus was produced in 4 weeks (Table-5). However the callus growth was fast in cultures having CM (Fig. 19B) and (Table-6).

The callus growing without CM was apparently compact and developed pink green, bright-white and hyaline areas in 4 weeks of culture. The callus growing with CM developed these colours one week later and it appeared as a loose growing mass. The green pigmentation was the dominant character of cultures growing without CM while the hyaline

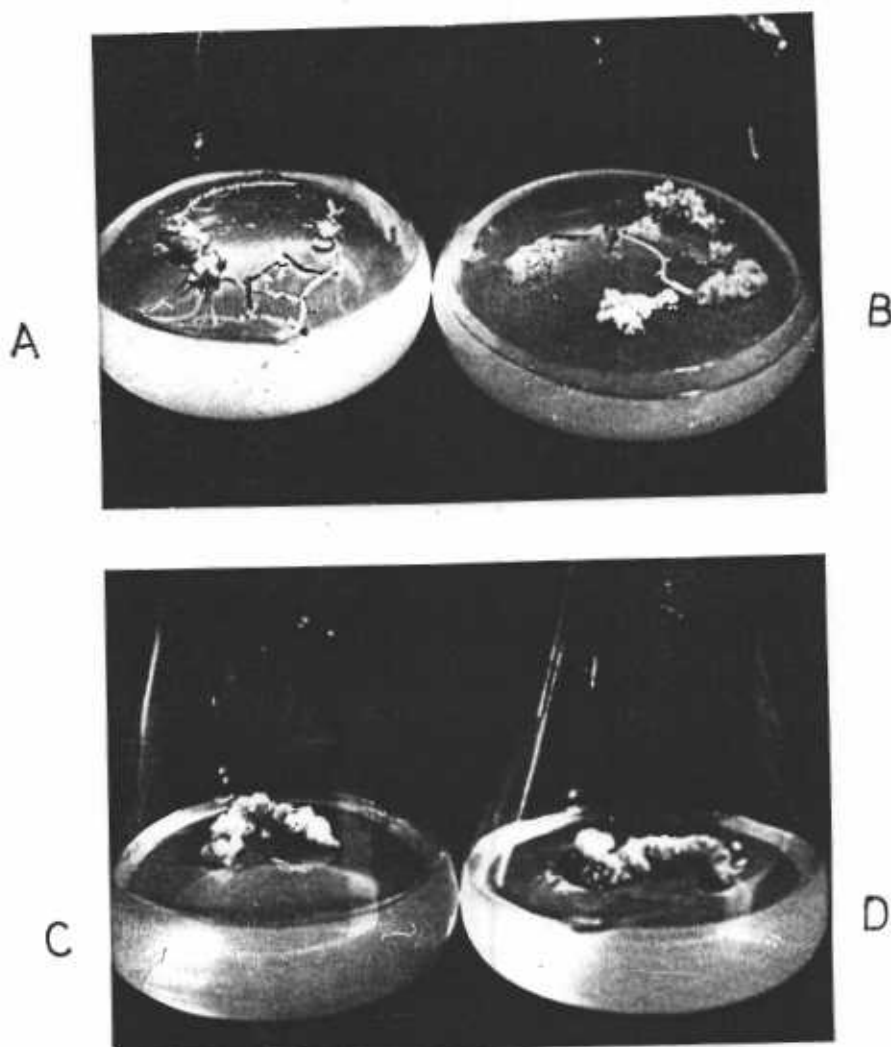


Fig. 19. Callus formation on *in vitro* differentiated root explants in 6 weeks cultured on White's Root Culture medium containing:

- (A) 0.1 mg/l NAA and 0.1 mg/l BAP
- (B) Treatment as (A) and 100 ml/l CM
- (C) and (D) 1 mg/l 2,4-D and 100 ml/l CM. (C) Sub-apical
- (D) Apical root segments.

callus for others. The fast growing CM callus when subcultured exhibited a gradual disappearance of the pink and green colours. Moreover it later developed off-white colour and grew for another 33 weeks, with only occasional appearance of hyaline areas.

b) Pigmented and bright-white callus

Pink, green and bright-white calli were isolated and subcultured as pure cultures on a medium with or without CM. In explants, cultured without CM, growth occurred in the second week, while in white and coloured calli in the third week. The white callus developed green tinge and the pink appeared in white calli. Similar pink areas also developed in cultures without CM. After 9 weeks, the actively growing white callus in tubes with CM and slowly growing green and pink calli were subcultured for another 6 weeks on a medium without CM. The transfer effected green pigmentation in white callus subcultures. Pink cultures became hyaline and green developed hyaline and white areas.

c) Hyaline callus.

The root explants were cultured on a medium containing 0.01 mg/l each of NAA and BAP and 0.1 mg/l of GA_3 . After about 4 weeks, a callus got initiated which was hyaline in appearance and grew for 6 weeks.

i) Effect of BAP and NAA.

Pieces taken from the above described hyaline callus were subcultured on media with or without CM. It started growth within 2 weeks in both the treatments. The hyaline callus was apparently granular in texture and became white in cultures with CM, while those without CM appeared somewhat lighter with greenish colour. After about 5 weeks, the pigmentation disappeared and the callus became all completely hyaline, in tubes with CM while those without CM sparsely developed a green tinge.

ii) Effect of 2,4-D and Adenine sulphate.

Hyaline callus was subcultured on either RC or MS medium containing 1.0 mg/l 2,4-D and 5.0 mg/l AS. Good growth was noticed after 3 weeks. Though the callus remained hyaline with a characteristic granular appearance, its growth was slower on MS medium in the following weeks (Fig. 20B). The callus growing on RC medium was then cultured with 0.1 mg/l NAA and BAP each. The callus exhibited more or less equally good growth similar to the cultures growing originally with NAA and BAP (Fig. 20C).

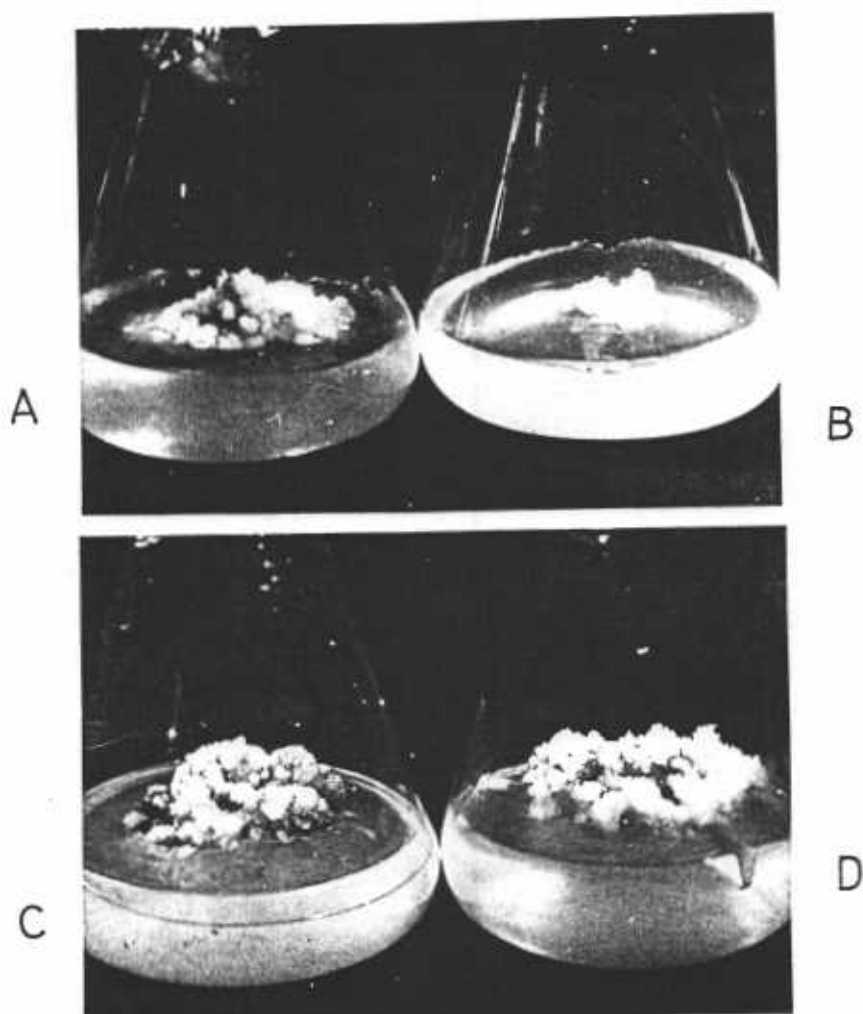


Fig. 20. Hyaline callus growing on:

- (A) White's Root Culture (RC) medium containing 1.0 mg/l 2,4-D, 5.0 mg/l AS and 100 ml/l CM (5 week old)
- (B) Treatment as in (A); medium MS (5 week old)
- (C) and (D) 0.1 mg/l NAA and 0.1 mg/l BAP and 100 ml/l CM in RC medium (8 week old); (C) Callus transferred from MS to RC medium.

IV-2. 2,4-D Induced Root Callus

a) Effect of CM

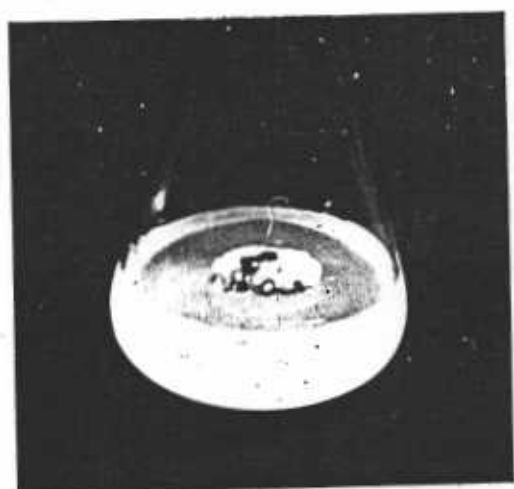
The root explants were sorted out into apical and sub-apical segments and both types were cultured on medium containing CM. The callus was formed in 3 weeks in all cultures which was more copious in apical segments (Fig.19D). The callus was apparently compact and greenish white in colour that became nodular.

In 15 weeks a white friable callus appeared and greenish-white compact callus changed to pure white compact callus. After 23 to 26 weeks of growth, hyaline green patches appeared within the callus, while pink colour changed to white mass. The growth of callus as such continued in the following weeks (Figs. 22 A (Upper) - C).

b) Effect of CH

Pieces of white compact, white friable and hyaline calli taken from CM containing flasks were subcultured separately on medium with CH.

Callus growth was noticed in 3 weeks and it was better in white compact and friable pieces (Figs. 21A-21C). Later the growth was found better for 6 weeks only in white compact callus.



A



B



C

Fig. 21. Root callus growing on White's Root Culture medium containing 1 mg/l 2,4-D and 1000 mg/l CH:

- (A) White and compact
- (B) Hyaline
- (C) White and friable.

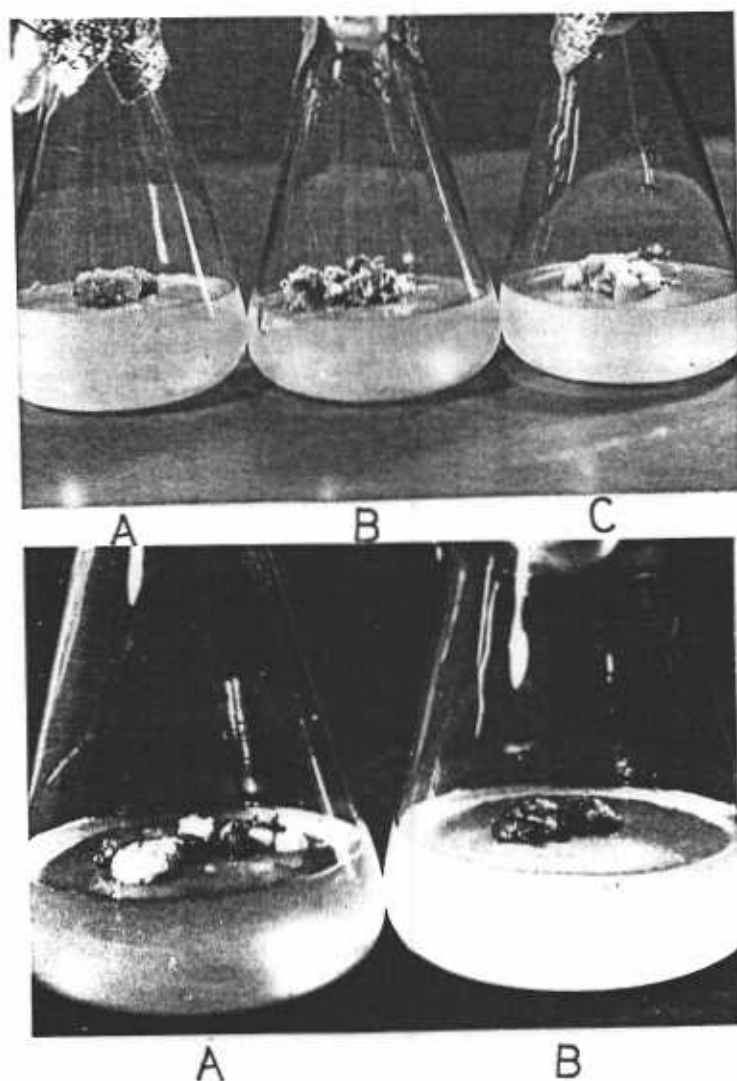


Fig. 22. Upper:- Root callus growing on White's Root Culture (RC) medium containing 1 mg/l 2,4-D and 100 ml/l CM:
 (A) Hyaline (B) White and friable (C) White and compact.

Lower:- Root callus showing growth in 3 weeks on medium containing 1 mg/l 2,4-D and 100 ml/l CM:
 (A) RC medium (B) MS medium.

c) White compact callus

White compact callus, growing on a medium with CM, was then subcultured on the same medium; it developed green pigmentation after about 26 weeks. Even that colour disappeared after two weeks, while the callus growth continued until 86th week.

The callus on transfer to MS medium containing CM, exhibited slow growth resulting in death of the callus after about 3 weeks (Fig. 22 B(Lower)).

V. BUDS CULTURE

These preliminary studies to culture the vegetative buds of *R. serpentina* were undertaken to achieve *in vitro* propagation. The buds taken from actively growing field plants were cultured on various media containing different growth regulators in addition to either 100 ml/l CM, or 500 mg/l CH, or 2000 mg/l YE or 10.0 mg/l biotin and extra nitrogen source (ENS) 250 mg/l SDDC. The pH of the media was adjusted to 5.6.

A) Culture of Buds on MS Medium

I. Effect of Kn in the presence of YE

The buds cultured on a medium containing 0.5 mg/l Kn sprouted in 2 weeks. The basal portion of buds swelled probably with the formation of internal callus. Growth of the shoot or initiation of root was not noticed in the following 6 weeks.

II. Effect of NAA with BAP or Kn.

The buds, cultured on a medium containing 1.0 mg/l NAA and 0.8 mg/l BAP, formed new leaves after 4 weeks, but in the following 7 weeks neither further shoot elongation nor root formation occurred. When transferred to a medium containing CM, the basal portion of buds however, swelled probably due to internal proliferations (Fig. 23B). These buds when transferred to a medium

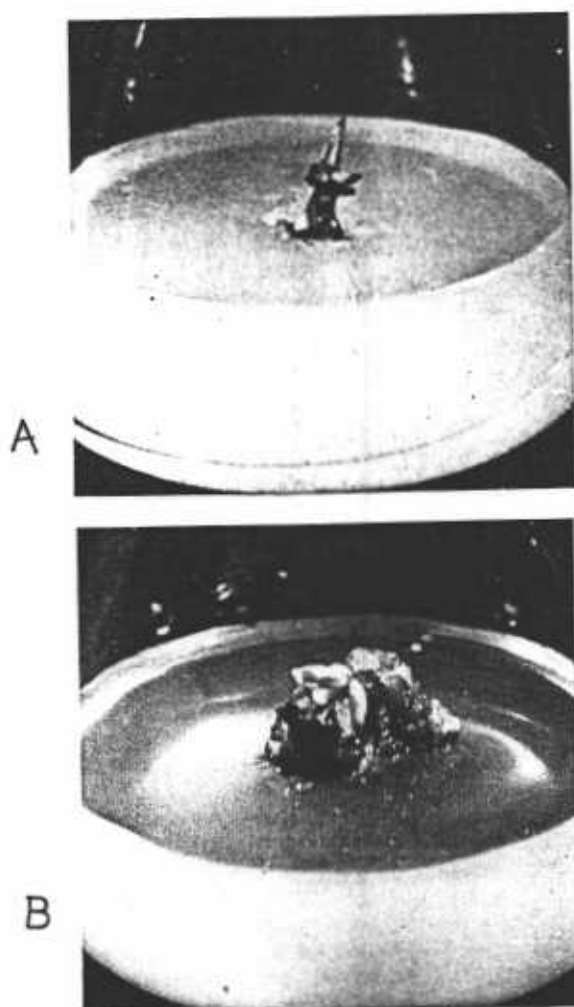


Fig. 23. Buds cultured on MS medium containing 100 ml/l CM and:
(A) 1.5 mg/l NAA and 15.0 mg/l Kn (2 weeks)
(B) 1.0 mg/l NAA and 0.8 mg/l BAP (8 weeks).

containing CH 1.0 mg/l NAA and 5.0 and 10.0 mg/l Kn, still failed to show any further development.

Some buds were cultured on a medium containing CM and with either 1.0 mg/l NAA and 10.0 mg/l BAP or 1.5 mg/l NAA and 15.0 mg/l Kn. The sprouting in all buds was visible in one week. The sprouting apparently was less in buds cultured with NAA and Kn. Furthermore, the basal portion of these buds grew in thickness as a result of internal proliferations (Fig. 23A).

B) Culture of Buds on AM Medium.

The buds were also cultured either on standard AM medium or the one containing only 1.0 mg/l NAA and 10.0 mg/l Kn. Sprouting of buds and the growth which followed in 8 weeks was similar to those buds cultured on MS media containing NAA and Kn, except that the buds on AM medium exhibited more vigorous growth.

C) Root Formation in Bud Culture.

The non-rooting buds were taken from MS and AM cultures and were soaked in liquid RC medium containing 3.0 mg/l 1AA and 1BA, for 24 hours and were then cultured on auxinless liquid RC medium. The root formation was observed

in 10 weeks and only in those buds which were comparatively less active in leaf production (Fig. 25A).

D) Culture of Buds on RC Medium.

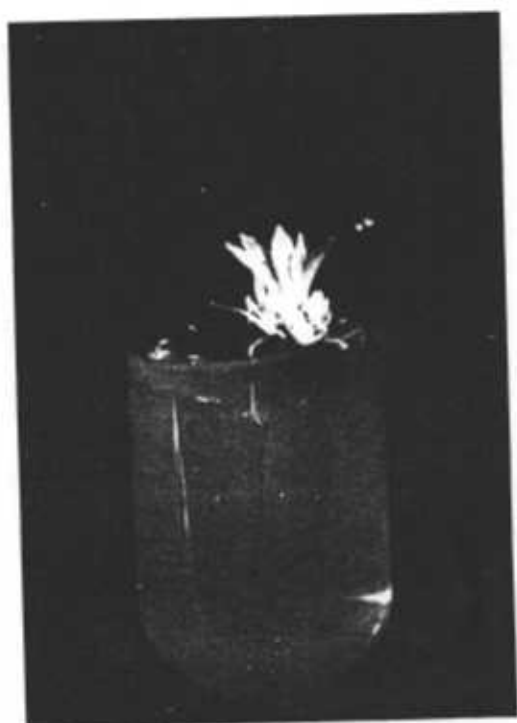
I. Effect of IAA and BAP

a) Buds with induced roots

The rooted buds were cultured on RC medium containing CM, 1.0 mg/l IAA and 10.0 mg/l BAP and biotin. In 2 weeks, the buds multiplied into 4 to 6 buds with new green leaves. Neither further growth of already induced root occurred nor were further roots induced within the observed period of 21 weeks in multiplied buds as well as in its sister buds when separated and cultured individually. The roottips and swollen basal portion of buds instead produced callus. The main axis of multiplied bud remained dwarf and shoot elongation was not noticed in the transferred cultures.

b) Buds without preformed roots.

The buds without prior root formation, when cultured on a medium containing CM, 1.0 mg/l IAA and 10.0 mg/l BAP and biotin, behaved in a similar way for 21 weeks as buds with preformed roots except that discoloration of leaf-tips occurred after 9 weeks. This discoloration was overcome by transferring the buds onto a medium containing 10 ml/l ENS solution.

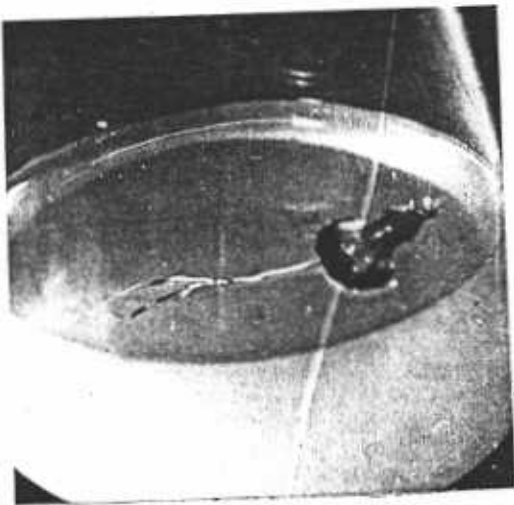


A



B

Fig. 24. Six week old bud culture on White's Root Culture medium containing 100 ml/l CM, 10 mg/l biotin and:
 (A) 1 mg/l IAA and 10 mg/l BAP
 (B) Bud growing without growth regulators.



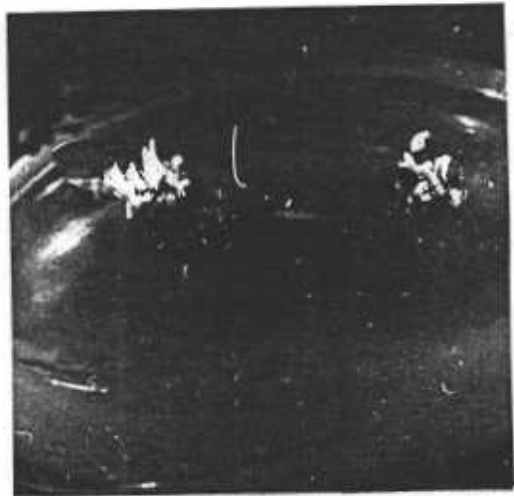
A



B



C



D

Fig. 25. Root formation in buds cultured on White's Root Culture (RC) medium with:

- (A) 3.0 mg/l IAA and 3.0 mg/l IBA (for 24 hrs. only)
- (B) Auxinless RC medium
- (C) Same as (B), root exposed
- (D) 0.8 mg/l NAA, 100 ml/l CM, 10 mg/l biotin and 250 mg/l Sod. diethyldithiocarbamate.

These non-rooting multiple buds were soaked for 24 hours in liquid media containing 3.0 mg/l of IAA and IBA each, followed by transfer to a liquid RC medium without any auxin. Instead of desired root formation, it was observed that in some multiple buds, a component sister bud became enlarged as if exhibiting shoot development (Fig. 24B). The leaves of enlarged buds were larger than those of other sister-buds and were of approximately 10 to 15 mm x 4 to 6 mm, in size. Further growth in the shoot stopped and these enlarged buds did not form roots (Table-9).

II. Effect of NAA

The buds were also cultured on a medium containing either only CM or with 0.8 mg/l NAA, 10.0 mg/l biotin, and SDDC. The buds were having yellowish green leaves and their basal portion produced a negligible amount of callus and roots in 13 weeks (Figs. 25B and 25C). The rooting was observed with NAA medium but after 2 more weeks (Fig. 25D). The rooting both with or without NAA was 10%.

III. Effect of BAP and GA₃

The rooted buds growing with or without 0.8 mg/l NAA were cultured on a medium containing CM, 10.0 mg/l biotin and

Table 9: Rooting and shoot development in cultured buds of *Rauwolfia serpentina* on White's Root Culture (RC) medium (without growth regulators). The buds were pre-treated with 3 mg/l IAA and 3 mg/l IBA for 24 hours in liquid RC medium.

Medium	Previous Culture		Buds growth behaviour (weeks)		
	Treatment (mg/l)	Sprouting initiation (days)	Multiplication	Shoot elongation	Rooting
MS	0.5 Kn+2000 YE	14	-	-	10
MS	1 NAA+0.8 BAP	28	-	-	10
MS	1 NAA+10 BAP+CM*	7	-	-	10
MS	1.5 NAA+15 Kn+CM*	7	-	-	10
AM	1 NAA+10 Kn+1000 CH	7	-	-	10
AM	1 NAA+0.3 Kn+0.1 BAP				
	+4 AS+6 2,4-D+1000 CH	7	-	-	10
RC	1 IAA+10 BAP+10 biotin				
	+CM**+ENS**	14	5	5	-

Legend:-

* concentration 100 ml/l

** concentration 10 ml/l.

ENS solution, 0.8 mg/l NAA, 2.0 mg/l BAP and 0.01 mg/l GA₃.

In 5 weeks, the roots swelled along the entire length, forming internal proliferations and new white coloured roots were produced on the old swelled and now completely blackened roots. No trend towards shoot development was however, observed in further 3 weeks.

VI. PLANTLETS FORMATION

For these studies, leaf, stem and root calli of *R. serpentina* were cultured on various media mentioned under respective experiments. The additives used were CM, SDDC, biotin and ENS solution and their concentrations are mentioned in the preceding results. The pH of the media was adjusted to 5.8.

The soil used for transplanting the plantlets was a mixture of loam soil and leaf mould. Its pH with tap water was 8.8. The pH of soil remained alkaline but varied to some extent with different solutions used for watering plantlets as referred to at appropriate places. The contrainers with transplanted plantlets were covered to increase inside humidity and were kept under the same conditions as provided to the culture flasks except for slight modification in illumination.

I) Suspension Cultures

A) Leaf Callus

For leaf suspension studies, the material came from cultures which had been actively growing on AM solidified medium for 6 weeks. Equal number of small callus pieces (cell aggregates) were cultured in flasks having AM suspension medium containing 100 ml/l CM. Growth in suspension cultures

and shoot differentiation was not noticed for the following 6 weeks after transfer.

B) Stem Callus

a) MS suspensions

For stem suspension studies on MS medium, the material came from cultures which had been actively growing on MS solidified medium containing 100 ml/l CM, 0.5 mg/l NAA and 1.0 mg/l BAP for 10 weeks. Equal number of cell aggregates were cultured in flasks having MS suspension medium containing 100 ml/l CM, 0.5 mg/l NAA, 1.0 mg/l BAP and 1000 mg/l CH. Growth in suspension culture on shoot differentiation was not observed for at least 6 weeks after transfer.

b) RC suspensions containing CM and Biotin

i) Effect of SDDC, CH and NAA

For stem suspension studies on RC medium, the material came from cultures which had been actively growing on RC solidified medium containing 100 ml/l CM, 10 mg/l biotin, 250 mg/l SDDC, and 0.8 mg/l NAA for 6 weeks. Equal number of cell aggregates were cultured in flasks having RC suspension medium, containing all the additives of solidified medium in addition to 1000 mg/l CH. Growth in suspension cultures was visible with minor increase in size of cell aggregates but without shoot differentiation after 9 weeks of culture.

ii) Effect of auxins and Cytokinins alone/or in combinations.

The stem callus was taken from actively growing callus cultures on RC medium containing 100 ml/l CM, 10 mg/l biotin, 250 mg/l SDDC and 0.8 mg/l NAA, during the last 23 weeks. Equal number of cell aggregates were cultured in flasks containing RC suspension medium, having 100 ml/l CM, 10 mg/l biotin and 1.0 mg/l IAA. The auxin ABA and cytokinins BAP, Kn, AS and 2iP were added to the suspensions, either alone or in various combinations (Table - 10). The suspension cultures exhibited some growth in all treatments upto 5 weeks. Thereafter, the suspensions stopped growth in most of the treatments and turned brown, except that a slow growth continued for about 15 weeks in cultures containing 10 mg/l of either AS or 2iP (Table-10). Moreover shoot differentiation was not noticed in these cultures for the period of 15 weeks.

C) Root Callus Culture

a) From *In vitro* differentiated roots.

For root suspension studies on differentiated roots, the material came from 40 weeks old cultures actively growing on RC medium containing 100 ml/l CM and 1.0 mg/l 2,4-D. Equal number of cell aggregates were cultured in flasks having RC suspension medium, containing 100 ml/l CM, 1.0 mg/l 2,4-D and 1000 mg/l CH. The growth in suspensions was noticed as cell

aggregates increased in size in 9 weeks. Shoot differentiation was not observed during this period.

b) From seedling roots

For root suspension studies on seedling roots, the material came from cultures which had been actively growing on AM solidified media for 19 weeks.

i) AM suspensions containing CM and IAA

Equal number of cell aggregates were cultured in flasks containing AM suspensions, having 100 ml/l CM, 2.0 mg/l IAA (in place of 1.0 mg/l NAA in standard AM) and half strength of other regulators of standard AM medium. The cells in suspensions neither browned nor exhibited any appreciable growth or differentiation for the following 9 weeks.

ii) Effect of dimethylaminopurine (2iP).

Cell aggregates were cultured with AM suspensions containing 100 ml/l CM, 2.0 mg/l IAA and half strength of AM growth regulators (excluding NAA). The cultures also contained either 0.16, 0.83, 1.6 or 8.3 mg/l 2iP. The cells did not turn brown. Shoot differentiation was not observed for 9 weeks.

2) Callus Cultures

For further studies on shoot differentiation only stem-callus cultures were utilized because in comparison

Table 10: Growth in stem cell aggregates of *Rauwolfia serpentina* suspended on White's Root Culture (RC) medium, supplemented with 100 ml/1 CM, 10 mg/1 biotin, 1 mg/1 IAA and different concentrations of ABA, BAP, Kn, AS and 2iP alone or in combination.

Treatment	Cell aggregates growth (weeks)			Remarks
	5	10	15	
0.01 ABA	+	+		Shoot differentiation was not observed in all treatments for 15 weeks.
0.05 ABA	+++	++		
0.1 ABA	+	+		
5 BAP	++	+		
10 BAP	++	+		
15 BAP	+	+		
5 Kn	++	+		
10 Kn	+			
15 Kn	+	+		
5 AS	+	+		
10 AS	+	+	+	
15 AS	+			
5 2iP	+			
10 2iP	+++	++	+	

Table 10: continued

Treatment (mg/l)	Cell aggregates growth (weeks)			Remarks
	5	10	15	
15 2iP	++			
2.5 BAP+2.5 Kn	+++			
5 BAP+5 Kn	+	+		
2.5 Kn+2.5 AS	++			
5 Kn+5 AS	+			
2.5 AS+2.5 BAP	+			
5 2iP+5 BAP	+			
1.5 BAP+1.5 Kn+1.5 AS	++			

Legend: =

+ negligible

++ fair

++ good

with leaf and root calli, they had more morphogenetic potential as expressed in root differentiation (page -63).

a) Cultures on MS medium containing CM.

i) Effect of NAA and Kn.

Stem callus initiated on MS medium containing 100 ml/l CM, 1.0 mg/l NAA and 0.5 mg/l Kn was subcultured on the same medium. No shoot differentiation was observed for 25 weeks after culture.

ii) Effect of NAA and BAP

The stem callus initiated on MS medium containing 100 ml/l CM, 1.0 mg/l NAA and 0.5 mg/l Kn was subcultured on MS medium containing 100 ml/l CM and 0.1 mg/l each of NAA and BAP for 60 weeks. The texture of the callus was generally friable but occasionally it assumed a nodular appearance with compact and hard texture. The changed texture was usually associated with appearance of green pigmentation. This green callus cultured with or without NAA and BAP, did not exhibit any shoot differentiation.

b) AM Cultures

The stem callus, previously growing on MS medium containing 100 ml/l CM, 0.5 mg/l NAA and 1.0 mg/l BAP, was subcultured on AM medium and grown for 30 weeks. It grew well, had friable texture and exhibited no shoot differentiation.

b) RC Cultures

The stem callus, previously growing on MS medium containing 100 ml/l CM, 1.0 mg/l NAA and 0.5 mg/l Kn, was subcultured on RC medium containing 100 ml/CM, 10 mg/l biotin, 250 mg/l SDDC and 0.8 mg/l NAA. This callus was subcultured regularly at 4-week intervals on media of the same composition. The callus grew for 20 weeks without shoot differentiation. Moreover, the callus, in the first few passages, remained nodular and compact. Thereafter, due to active growth, the new callus on the periphery of the callus inoculums became friable. However, the callus in the inner core, base and at the slow growing portions of the inoculum remained hard and nodular. After the 5th passage (i.e. 22nd week) spontaneous occurrence of meristemoid structures was noticed in cultures. Their appearance was like thick and blunt white projections. A low percentage of these white meristemoids however, grew further and formed well growing green shoots (Fig. 26(Upper)) within 2 to 4 weeks. Once established in culture, these shoots took a further 4 to 6 weeks period for root formation.

After root formation, the plantlets could easily be separated from the mother callus (Fig. 26(Lower)). The plantlets containing 100 ml/l CM, 10 mg/l biotin, 1.0 mg/l IAA and 10.0 mg/l BAP. After four weeks, growth in apical bud was noticed. Later, the leaves became yellowish, the roots formed callus and new shoots grew out from the basal nodes (Fig. 27(Upper)).

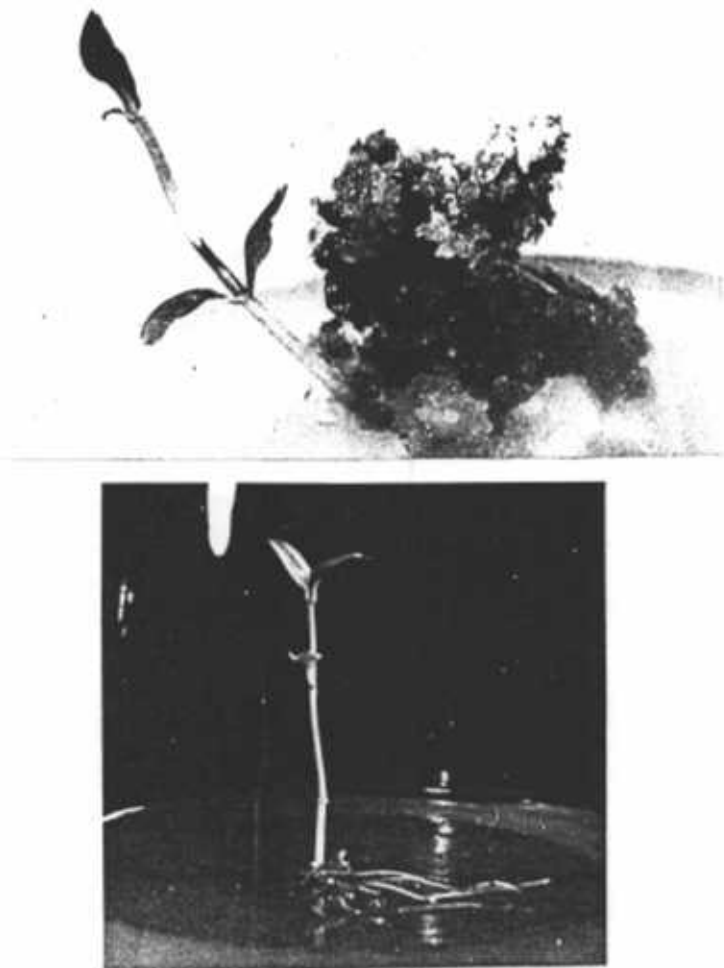


Fig. 26. Upper:- Shoot differentiation in stem callus cultured on White's Root Culture medium containing 100 ml/l CM, 10 mg/l biotin, 250 mg/l Sod. diethyldithiocarbamate and 0.8 mg/l NAA.

Lower:- Shoot differentiated in stem callus, cultured as above showing root formation in 4 weeks.

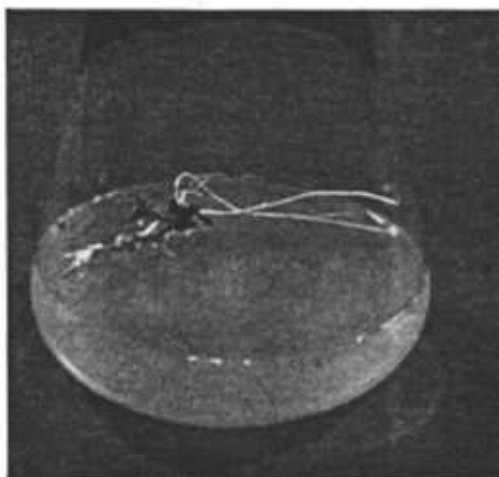


Fig. 27. Upper:- Callus formation on plantlet's root on White's Root Culture medium containing 100 ml/l CM, 10 mg/l biotin, 1 mg/l NAA and 10 mg/l BAP.

Lower:- Rooting of plantlets in basal RC medium, after treatment for 24 hrs., with 3 mg/l IAA and 3 mg/l IBA. (Callused root was excised).

With the further development of new shoots, the growth of apical buds slowed down. To eliminate or reduce yellowing of leaves, the medium was supplemented with 10 ml/l ENS solution in further subcultures.

In the following 5 weeks, profuse callus formed on the plantlet roots and new shoots grew out and formed branches with more green leaves. The callused roots were separated from the plantlets and the plantlets now without roots, were cultured on medium containing 100 ml/l CM, 10 ml/l ENS solution, 10 mg/l biotin, 0.8 mg/l NAA and 2.0 mg/l BAP.

The plantlets did not produce any root in the following 9 weeks, but their swollen basal portion, ruptured and produced green callus which apparently was the extension of internal callus or proliferations. Nonetheless the shoots exhibited vigorous growth. The callus formed at the basal end of the plantlets was separated and the plantlets were then treated with 3.0 mg/l IAA and IBA each for 24 hours in liquid RC medium. Thereafter, the plantlets on transfer to auxinless liquid RC medium formed roots after one week. The roots were allowed to enlarge for 14 days (Fig.27 (Lower)0, before culturing the plantlets on solidified RC medium containing 100 ml/l CM, 10 ml/l ENS solution, 10 mg/l biotin, 0.8 mg/l NAA, 2.0 mg/l BAP and 0.0001 mg/l GA_3 . After 6 weeks the plantlets showed slow shoot growth which, however, formed new green leaves. The root tips formed callus but no new roots were formed. In further transfers of plantlets on

fresh medium, the concentration of GA_3 was increased to 0.001 mg/l. In the following 13 weeks, the root of plantlets produced copious green callus (Fig. 28). The branched shoots formed more green leaves. The older leaves turned brown and abscised almost like field growing plants.

The callused roots were then separated from the plantlets. The plantlets were retreated with IAA and IBA for 24 hours and were then cultured on auxinless liquid RC medium for re-rooting. The plantlets produced roots as before, in auxinless liquid RC medium. The rooted plantlets were left for further development of roots for the following 2 weeks and were then transferred in soil and provided with extra illumination (tungsten light) as 100 W per 5 plantlets. The plantlets were watered with tap-water. The pH of soil after watering was 8.3.

In the following 2 weeks, the transplanted plantlets gave normal and healthy appearance similar to the field plants. Leaves were mostly green but some were slightly yellowish at tips. Some growth in the apical buds was noticed (Fig. 29). The leaves at the lower nodes were abscising with sprouting of new leaf-buds. This condition, however, could not be prolonged beyond one week when the tips of leaves became yellow and turned downwards and ultimately such leaves started falling off. The tap water was thereafter mixed with 10.0 ml/l ENS solution, by doing so the pH of the medium became 8.6. Even the addition of ENS could not check leaf abscission and the situation did not



Fig. 28. Growth of plantlet on White's Root Culture medium supplemented with 100 ml/l CM, 10 mg/l biotin, 10 ml/l ENS (Extra nitrogen source), 0.8 mg/l NAA and 0.001 mg/l GA_3 .

improve in 2 weeks. The plantlets were provided with macro-salts of RC medium and boric acid, manganese chloride, zinc sulphate, potassium iodide and Fe EDTA in standard concentration in solution form. Final volume of this nutrient solution was adjusted with tap-water. ENS solution as 10.0 ml/l was also added to this nutrient solution. The pH of the soil with this solution remained 8.6. With this nutrient solution, plantlets regained their vigour in one week. The leaves became green but the slight yellowishness of tips still remained. However, further falling off of the leaves stopped. The new leaves were completely green and the growth in apical buds was improved. The older leaves later acquired complete yellow colour. The nutrient solution was changed with the one containing only the macro-salts of RC medium in tap water and 10 ml/l ENS solution. The pH of soil with the new solution became 8.5. After the application of changed nutrient solution leaves near apical buds seemed protected from further yellowing, but in the following 2 weeks the midribs and veins of leaves were seen becoming yellow, otherwise the leaves were healthy. Growth in plantlets, however, continued at slow pace. Thereafter, the plantlets were given half strength Knop's solutions, containing micro-salts of Berthelot's solution. The pH of soil with this solution became 8.1. The tungsten light illumination was doubled (200w) (Table-11).

The plantlet leaves acquired complete green colour in the following 2 weeks. They were growing in 225 mm dia., pots,



Fig. 29. *In vitro* raised plantlet after 2 weeks of transfer in the soil.

flooded with 100 ml nutrient solution every 72 hours. The plantlets attained average size of 70 mm after 10 weeks in the soil.

Table 11: Effect of varied intensity of illumination on regenerated plantlets of *Rawolfia serpentina* growing in potted soil, under 28°C $\pm$ 1 temperature and 16/8 hours light/dark condition (Humidity was maintained at 90%).

Fluorescent Tubes (W)	Illumination intensity		Period duration (days)	Leaf Colour
	Tungsten lamps(W)	Total light(Lux)		
80	100	1206	35	Leaves green except slight yellowing of tips and occasional appearance of yellow colour to midrib and veins.
80	200	1230	30	Leaves completely green in colour.
100	800	1318	4	Leaves became lemon yellow.
100	-	1172	4	High intensity illumination* injury recovered; all the effected leaves regaining green colour.

Legend:-

W = Watts

* = Tungsten

DISCUSSION

From the results described in the preceding chapter it is evident that the callus tissue induced from leaf, stem and root segments of *Rauwolfia serpentina* could be successfully and continuously cultured. Further culturing of stem callus resulted in root and bud differentiation, ultimately leading to plantlet formation.

A) Callus Formation and its Culture

a) Effect of explant origin.

In the present studies, the formation of callus was observed better and earlier on leaf explants taken from laboratory grown aseptic seedlings than from field plants. Mitra *et al.* (1965) reported that in leaf explants, taken from aseptically grown seedlings of *Rauwolfia serpentina*, callus formation was confined to the vascular regions, particularly those of mid and lateral-veins. Likewise Furuya *et al.* (1972 and 1974) obtained petiolar callus in *Coptis japonica* var. *dissecta* and *Rheum palmatum*.

Stem explants taken from field growing plants behaved normal and without any mentionable difficulty in callus formation.

Mitra *et al.* (1965) also reported formation of root callus in sub-apical explants taken either from the main

or lateral roots. In contrast to their observations the present studies showed that both the apical (root-tips) and sub-apical root segments formed copious callus. The root segments taken from aseptic seedlings and from roots differentiated in stem callus, were found better explants for callus formation than roots of field grown plants.

b) Effect of various media

For the induction and growth of leaf callus of *Rauwolfia serpentina*, White's, MS (Mitra *et al.* 1965) and Knop's (Perveen, 1978) media had been used with similar results. Furthermore, Perveen (1978) reported that the basal medium supplemented with coconut milk and auxins induced callus in segments of leaf and stem. The isolated callus tissues could not grow continuously by regular subculturing at one month's interval in the same medium in which they were induced. Mitra *et al.* (1965) suggested that after induction of callus on White's medium MS was a suitable medium for successful continuous growth of leaf and stem callus tissues. In these investigations leaf callus was induced on MS medium and its successful continuous growth was achieved on AM (Abou-Mandour 1977) medium.

MS, modified White's (Mitra *et al.*, 1965) and Knop's medium (Perveen, 1978) were also used for the induction and growth of stem callus. In the present work, MS medium was found suitable for callus formation on stem explants and modified

White's root culture (RC) medium (Thomas and Davey, 1975) suitable for continuous culture of stem callus. The stem callus usually became brownish in long duration cultures on MS medium, while browning was not as frequent on modified White's (RC) medium. Similarly Carew (1966) was able to maintain callus cultures of *Catharanthus roseus* for more than 3 years on another modification of White's medium (Babcock and Carew, 1962). These results are, however, different than generally reported for MS medium, which is in vogue for culturing callus on agar and is cherished due to its high content of nitrate, potassium and ammonium relative to other nutrient media (Gamborg *et al.*, 1976). Moreover, the amounts, particularly of nitrogen and potassium, have been found to be inadequate in White's medium to sustain maximum growth of callus (Murashige and Skoog, 1962). These results also differ from the observations made by Perven and Ilahi (1978), who transferred the stem callus for continuous growth, from a low salt medium (Knop's) to a high salt medium (MS).

In this work, root callus formation and continuous culture of root callus was achieved on modified White's (RC) medium. Other workers also obtained callus in explants of *R. serpentina* roots on a modified White's medium (Mitra and Kaul, 1964). The *in vitro* differentiated roots from stem callus of *R. serpentina* formed callus on modified White's (RC) medium. Moreover, these callus cultures were successfully and continuously maintained on the same medium during the present studies.

c) Effect of auxins or cytokinins alone and in combination.

In the present work, 2,4-D alone was found considerably effective for callus formation in leaf, stem and root segments at various concentrations ranging from 0.1-1.0 mg/l. The leaf and root segments required a comparatively higher concentration (1.0 mg/l) than the stem explants.

Perveen (1978) also observed that leaf explants of *R. serpentina* required a higher concentration of auxins for callus formation than stem segments and that the callus could also be induced in leaf segments at a low auxin level (0.1 mg/l) when combined with high level of cytokinin (Kn, 5.0 and 10.0 mg/l).

Nonetheless, 2,4-D used alone proved better auxin than NAA for callus induction in leaf, stem and root explants. These results are in agreement with various workers who found 2,4-D alone as an effective auxin, mostly at the concentration of 1.0 mg/l (Staba, 1969) or occasionally at a higher concentration, 3.0 mg/l (Carew, 1966). Mitra *et al.* (1965) also found 2,4-D alone as an essential component with White's and MS media for continuous growth of leaf, stem and root callus cultures of *R. serpentina*. In the present work, a cytokinin was not usually required with 2,4-D for continuous culture of stem and root calli. In fact the addition of Kn to cultures in combination with 2,4-D was found inhibitory to stem callus growth. Hosoda and Yatazawa (1979) observed similar effect of kinetin on *Solanum laciniatum* cultures. In the present investigations

adenine sulphate (AS), kinetin (Kn) and benzylaminopurine (BAP) had useful combined effect (total cytokinins=4.4 mg/l) with a high concentration of 2,4-D (6.0 mg/l) in AM medium for successful and continuous culture of leaf callus. Perveen (1978) did not get the similar combined effect of 2,4-D with a cytokinin (Kn) for callus formation and callus growth in leaf and stem explants of *R. serpentina*. The results of present investigation, however, concur with the results of various workers who reported the combined effect of 2,4-D with Kn (Dhoot and Henshaw, 1977), BAP (Javeed *et al.*, 1980), Kn, BAP and AS (Abou-Mandour, 1977) BA (Zain-ul-Abedin, 1977) on callus formation.

Besides 2,4-D, Absciscic Acid (ABA) was also used for cell growth in suspension cultures and it enhanced the growth in the presence of IAA. The results are in agreement with Minocha (1979); who found that ABA stimulated the growth by cell division in combination with an auxin (NAA) in *Helianthus tuberosus* tissue.

NAA, mostly helped in the presence of a cytokinin. A high concentration of cytokinin (Kinetin, 10.0 mg/l) was suggested by Perveen (1978) with NAA (1.0 mg/l) for formation of callus in leaf explants. In the present investigation it was observed that with NAA, the presence of a cytokinin (Kn or BAP) enhanced the callus formation and the growth of callus was found better. In leaf explants, a high concentration of

cytokinin (Kn, 10.0 mg/l) was required with NAA (1.0 mg/l) for callus formation.

The stem callus growth was found better in cultures containing Kn with NAA in the medium (1.0 mg/l each). The results are in agreement with Ramawat *et al.* (1978) for *Catharanthus roseus* stem callus cultures.

The root segments required similar dose of 2,4-D as by leaf segments (1.0 mg/l). However NAA and a cytokinin (BAP) when used together was found sufficient to form callus and to continue its growth even at lower concentrations (0.1 mg/l). In *Digitalis purpurea*, 2,4-D also induced better callus at a lower concentration (2.0 mg/l) and NAA at a much higher dose (10.0 mg/l) (Staba, 1962). The *in vitro* differentiated roots behaved similar to seedling roots regarding effect of auxins and cytokinin, for callus formation and culture in the present studies.

d) Effect of organic supplements in culture medium.

Coconut milk (CM) 10-15% (Mitra *et al.* 1965), casein hydrolysate (CH) 500 mg/l (Perveen, 1978) and yeast extract (YE) 0.1-0.2% (Ohta and Yatazawa, 1979) had been used for formation and culture of callus in *R. serpentina*. In the present studies CM used as 10% (v/v) in MS medium was found suitable for callus formation in leaf segments as well as for initiation and continuous growth of stem callus and in modified White's (RC) medium for root callus. CH, at 1000 mg/l, was

found suitable for continuous growth of leaf callus. YE, used as 0.1-0.2% with stem callus cultures, did not improve the callus growth on MS medium.

Most plant cells, in general, grew well without these organic supplements, provided the salt concentration of the culture medium was sufficiently high (Gamborg and Wetter, 1975). In the present work, CH enhanced leaf callus growth in a high salt medium (AM) and CM worked well both for stem and root callus cultures on high (MS) and low salt, White's (RC) medium respectively.

Gamborg *et al.* (1976) suggested that thiamine (Vit.B) alone may be sufficient to culture most of the calli. In their studies, Nicotinic acid (Vit. B) and pyridoxine (Vit.B₆) were present with thiamine in MS, modified White's (RC) and AM media used for the culture of leaf, stem and root calli of *R. serpentina*. In the present investigations, the stem calli grew better with the addition of biotin (Vit.H) in the culture medium.

Sucrose was used as a carbohydrate source in the culture media, during the present investigations. At lower sucrose levels, cf 1.5 in White's and 2.0% in MS medium, occurrence of pigmentation (mostly green) was frequent in light exposed cultures of stem and in root callus with BAP and/or NAA.

The pigmentation was rarely observed in 2,4-D root calli growing on similar sucrose concentration. Bergmann (1967) also observed that auxin, 2,4-D, adversely affected chloroplast induction in cultures.

Relative abundance of pigments was more in a medium supplemented with 1.5% sucrose than in one containing 2.0%. Pigmentation was never observed with AM medium containing 3% sucrose. Sucrose concentration higher than 1.5% might be inhibitory for pigment production in *R. serpentina* callus cultures. Dalton and Street (1977) and Dalton (1980), also noticed that 2.5 mg/ml or higher concentration of sucrose was unfavourable to chlorophyll synthesis in *Spinacia oleracea* cultures. On the other hand, Fadia and Mehta (1976) reported increase in chlorophyll formation with increasing levels of sucrose from 0.5 to 4.0% in *Cucumis melo* callus cultures.

In the present studies, occurrence of pigmentation or reversion to a nonpigmented state in callus cultures was a common observation. This behaviour agrees with the results obtained by Straus (1958) for corn endosperm tissue cultures.

e) Effect of cultural conditions.

Following the recommendations of Mitra and Kaul (1964), the *Rauwolfia* cultures were kept at a constant temperature of $28^{\circ}\text{C} \pm 1$ which was found suitable for continuous growth of

leaf, stem and root calli. The initial pH of various media adjusted to 5.8 was also in accordance with other workers recommendations (Gamborg *et al.*, 1976; Ohta and Yatazwa 1979). The 16 hours illumination provided to cultures in general, was found suitable for callus formation and callus culture, from stem and root segments. The leaf callus was, however, induced more easily in continuous illumination and thereafter exhibited equally good growth pattern similar to stem and root calli in 16 hours illumination. Dark grown cultures gave soft and friable stem callus similar to that obtained by Perveen (1978) under similar conditions.

B) Root Differentiation and Root Culture

a) Effect of callus origin, texture and pigmentation

In the present work, root differentiation was not observed in leaf callus. The roots were observed in root callus but after a prolonged culture of 11 weeks. In contrast, the stem calli frequently differentiated roots. In general, the compact and hard stem calli formed roots. When the compactness was changed, as by transfer of cultures to CM containing media, the root differentiation capability diminished almost parallel to the degree of compactness.

Pigments favoured root differentiation only to a limited extent. In contrast, milky-white stem callus was

found best for early root differentiation. In the same culture rooting was concomitant to the callus growth.

b) Effect of pH and illumination

Root formation was observed in light exposed cultures in both pigmented and non-pigmented calli. Most of the pigmented calli differentiating roots remained so only for a short duration. Thereafter they gradually reverted back to non-pigmented state. Vasil and Hildebrandt (1966) observed better root formation in petiolar callus of *Petroselinum hortense* in the dark. Moreover, illumination was also found necessary for the successful culture of the roots in the present studies.

The root differentiation occurred at 5.8 pH, while Ohta and Yatazawa (1979) reported differentiation of roots from root callus of *R. serpentina* at 6.0 pH.

c) Effect of media, vitamins, SDDC and Coconut milk.

Root differentiation occurred on both MS and White's media but was more frequent and continued for a longer time on the later. Stem callus growing on AM medium did not differentiate at all.

Root cultures grew successfully only on White's medium. The absence of ammonium nitrate may be one of the reasons as Mitra (1968) reported that ammonium nitrate, even

in very low concentration (30 mg/l), was not beneficial to the growth of excised roots. Besides B-vitamins (thiamine, pyridoxine and nicotinic acid), vitamin H (biotin) used in White's medium was found effective in root differentiation for longer periods (59-84 weeks). Biotin in the same concentration (10.0 mg/l) was also found suitable for root culture. It helped in early initiation of laterals on root segments. SDDC (250 mg/l) was added to stop browning in stem calli. The browning of calli negatively affected the root-differentiation and with the addition of SDDC the restoration of growth in calli resulted in the increased root differentiation.

Roots in culture formed lateral roots more favourably when cultured with 100 ml/l coconut milk (CM). The average increase was 110.86% as compared to 15.83% for roots cultured without CM. Moreover when the callus was left attached with the cultured roots, white callus favoured lateral root formation better (110.86%) than green callus (42.65%) when cultured with coconut milk.

d) Effect of growth regulators

The root callus of *R. serpentina*, in contrast to stem callus, differentiated roots with 1 mg/l 2,4-D. The results obtained with root callus are of particular interest as 2,4-D is generally believed to repress morphogenesis in plant cultures (Gamborg *et al.* 1976).

The auxin, NAA, was found to induce roots in stem callus of *R. serpentina* in the present investigation at concentrations varying from 0.1-0.8 mg/l. At 0.8 mg/l and higher concentration, callus was induced on explants of differentiated root. Callusing of excised roots of *R. serpentina* was also obtained by Mitra (1968) with 1 mg/l NAA.

In *Pelargonium hortorum* cultures, root formation was noticed with 0.01-1.0 mg/l NAA (Narayana, 1963). While, *Catharanthus roseus* required higher concentration of NAA (1-10 ppm) for root formation (Ramawat *et al.*, 1978). The differences may be attributed to the nature of the material used.

Cytokinin (BAP) was effective in root formation as well as in culturing the differentiated roots. Kinetin was equally effective in root differentiation in the present studies. Ohta and Yatazawa (1979) reported root formation in *R. serpentina* cultures with 0.2 ppm kinetin and 1 ppm 2,4-D. In contrast, Perveen (1978) reported that exogenous application of cytokinins was deleterious for the initiation and elongation of roots, in *R. serpentina*.

In *Petroselinum hortense* tissue culture, an inhibition of root formation was noticed by increasing the concentration of auxin (IAA, at 1, 2.5, 5 and 10.0 mg/l). Root formation was, however, suppressed with higher concentrations of kinetin (2.0-8.0 mg/l) in *Pelargonium hortorum* (Narayana, 1963).

In the present work, cytokinin (BAP) and NAA used in combination at lower concentration (0.1 mg/l) and NAA alone at higher concentration (0.8 mg/l) were found equally good both for root formation in callus culture and for culture of differentiated roots. The results obtained with equal concentrations of auxin and cytokinin varied from those of Skoog and Miller (1957), who reported that higher relative concentrations of auxin to kinetin promoted root growth in tobacco callus.

In the present studies higher concentrations of NAA used alone (0.5-0.8 mg/l) or a low concentration (0.1 mg/l), combined with equal dose of BAP, were found equally effective for culture of differentiated roots. These results, in part, supported the findings of Mitra (1968), who obtained laterals formation in excised roots of *R. serpentina* at optimal concentration of 0.8 mg/l NAA. Although he reported that cytokinin (Kn) at 0.001-0.01 mg/l concentrations was deleterious to root growth.

C. In vitro Propagation

In vitro propagation of *R. serpentina* is possible either by root induction in cultured shoot-tips or by formation of plantlets through bud differentiation in callus cultures. The efforts for embryogenesis in cell cultures so far have not been successful.

The buds cultured on MS medium containing either 0.5 mg/l Kn alone or 1.0 mg/l NAA and 10.0 mg/l BAP, swelled at the base probably due to internal proliferations. These buds did not produce roots either on MS medium or on auxinless liquid RC medium. The rooting technique of Gupta *et al.* (1980) reported for woody plant species (e.g. *Tectona grandis*) did not work favourably for *R. serpentina* cultured buds. The culture of buds in auxinless medium favoured multiple buds production. The multiple bud-formation was also achieved on White's medium containing 1.0 mg/l IAA and 10.0 mg/l BAP. The rooting of these buds was achieved on White's medium with or without 0.8 mg/l NAA. The percentage of rooting was very low which occurred only after culturing these buds for a long period of 10 weeks. The results agree in part with Mitra (1976b), who obtained multiple buds in cultured apical shoots of *R. serpentina*, on medium containing 1.0 mg/l NAA and 0.8 mg/l BAP. This method of vegetative propagation was unsuccessful because of slow rooting of buds and lack of shoot formation by rooted buds.

The bud differentiation in callus cultures of woody plant species does not occur so readily (Thorpe, 1977 and 1979). The majority of reports on bud regeneration are, therefore, met within herbaceous plant species or in crop plants.

In the present studies, the bud regeneration did occur in stem callus. These results agree with Heble and

Chadha (1978) who reported morphogenetic potential in *Hemidesmus indicus* stem callus and none in root callus cultures. The shoot differentiation in root cultures and morphogenetic potential in leaf cultures of *R. serpentina* were not observed in the present work. The results differ from Mitra and Chaturvedi (1970) who obtained shoot differentiation in leaf callus of *R. serpentina*. The reasons for not obtaining differentiation, may be the higher concentration of 2,4-D (6.0 mg/l) in AM medium. The leaf callus culture in the present work required AM medium for their vigorous growth. Three cytokinins in the total concentration of 4.4 mg/l could not revert the suspected inhibitory effect of 2,4-D. Furthermore, the root cells in suspension medium with lesser concentration of 2,4-D (1.0 mg/l) or the stem callus at a much low concentration of ABA (0.01) showed no shoot differentiation. Staba (1969) also got similar results and suggested that at higher concentration, 2,4-D may adversely affect morphogenesis. But such a suggestion may not be tenable because Hosoda and Yatazawa (1979) did not obtain shoot regeneration even at low concentrations (0.1 ppm) of 2,4-D in *Solanum laciniatum* cultures. The results in these investigation, however, differ from those of various other workers who obtained shoot regeneration with 2,4-D, at various concentrations (0.1-100 mg/l), when combined with different cytokinins such as with 5.0 mg/l Adenine in *Tylophora indica* cultures (Rao *et al.*, 1970); 2iP

(isopentyle adenine) 3 mg/l in *Phoenix dactylifera* (Tisserat, 1981) and 0.5 mg/l kinetin in *Saccharum officinarum*, (Narayana and Sinivasan, 1971). In the callus cultures of *Medicago sativa* (Saunders and Bingham, 1975), the frequency of bud formation was directly proportional to the 2,4-D concentration in the range of 2.3-54 μ M.

During the present work, the shoot regeneration in stem callus cultures was obtained on modified White's (RC) medium which was also suitable for further development of plantlets. The results varied from crop plants as sugarcane (Heinz *et al.*, 1969), corn (Green and Phillips, 1975), oat (Cummings *et al.*, 1976), sorghum (Gamborg *et al.*, 1977) where the basic medium for *in vitro* morphogenetic studies predominantly was MS.

Calli of *Lycopersicon esculentum* (Herman and Haas, 1978), which had undergone one or repeated subcultures without shoot formation, could not be induced to form shoots. In contrast, during the present investigations, the stem callus of *R. serpentina* regenerated shoot buds only after a subculture period of 20 weeks. The results agree with Bajaj *et al.* (1973) who obtained shoot regeneration in callus cultures of *Sinapis alba*, 20 to 25 weeks after first subculture; 52 weeks in *Catharanthus roseus* (Abou-Mandour *et al.* 1979) and 2 years in *Populus tremuloides* (Winton, 1970).

Organic supplement as CH, 500 mg/l or YE, 200 mg/l in the presence of 10% CM were found essential for shoot regeneration in *R. serpentina* callus cultures (Mitra and Chaturvedi 1970) and also by 100 ml/l CM in the present work.

NAA at 0.8 mg/l induced shoot regeneration in stem callus of *R. serpentina*. The results agree in part with Pillai and Hildebrandt (1968b) and Raj Bhansali *et al.* (1978), who found NAA at 0.1 mg/l and 0.25 mg/l best for shoot regeneration in *Pelargonium hortorum* and *Crotolaria burhia* respectively.

For shoot regeneration in *R. serpentina*, the culturing of callus on the same media composition including regulators was necessary for a certain period (20 weeks at least). The results agree with Abou-Mandour *et al.* (1979), who cultured *Catharanthus roseus* callus in a similar way programmed for 52 weeks for shoot regeneration.

The stem callus of *R. serpentina*, which regenerated buds, was heterogenous in texture, being friable at the periphery and top but compact and hard at the base and core. Shoot regeneration in *R. serpentina* from the basal part of inoculated callus piece was similar to observations reported by Ross *et al.* (1973) for tobacco callus.

Plantlets in *R. serpentina* cultures also developed

roots on the media which generated the shoots. The plantlets were subsequently transferred to medium with high cytokinin (BAP, 10 mg/l) for further development. A major problem with the development of plantlets was the formation of callus on their roots. The roots forming callus ceased its normal development which was otherwise necessary for transfer of plantlets to soil. If the callused roots were separated from the plantlets and these plantlets were rooted by using IAA and IBA (Gupta *et al.*, 1980), the induced roots were again found heavily covered with new callus.

As suggested by Morel (1964), GA₃ (0.0001 mg/l or 0.001 mg/l) used in the medium to check the callusing of roots was not effective. Further higher doses of GA₃ were avoided as at 10 mg/l, it was found strongly inhibiting shoot formation in tobacco and instead enhanced callus growth (Mura-shige, 1964). For transfer of plantlets to soil, their root development was achieved in auxinless liquid White's medium.

The leaf shedding of field growing plants was complete at the time of transfer of regenerated plantlets to soil in October; it was the main reason that the plantlets were potted and kept at 28°C in incubator. As the incubator conditions differ in many respects (e.g. day and night temperature; illumination intensity; seasonal variations and so on) from the conditions under which the plant grew in nature, therefore, the slow growth was expected. The various nutrient

solutions tested, were from simple tap water to ENS solution, modifications of macro-and micro-salts of White's (RC) medium, terminated at half strength Knop's solution with Berthelot's solution for micro-elements. The preference by plantlets for a simple nutrient solution may partly be due to their ability to manufacture their own food by photosynthesis.

Plantlet's requirement for light necessiated use of a small source of tungsten (100 - 200 W) alongwith fluorescent illumination. However with high tungsten illumination (800 W), the leaves were injured, which later on acquired healthy appearance and green colour when the tungsten light was removed.

SUMMARY

For callus formation, the leaf explants of *R. serpentina*, obtained both from field growing plants and from the aseptic seedlings were cultured with 2,4-D and NAA in various combinations. Copious callus formation was obtained on explants taken from aseptic seedlings with 1 mg/l 2,4-D and 1 mg/l NAA combined with 10 mg/l Kn, on MS medium. Leaf callus from aseptic seedlings grew favourably on AM medium with its standard concentration of growth regulators and 1000 mg/l casein hydrolysate (CH).

The highest incidence of callus formation occurred on ^{stem} explants obtained from field growing plants, with 1 mg/l 2,4-D and 1 mg/l NAA added singly to MS medium. Cultures of stem callus showed copious growth with 0.8 mg/l NAA used with 100 ml/l coconut milk (CM), 10 mg/l biotin and 250 mg/l sodium diethyldithiocarbamate (SDDC) or with 0.1 mg/l NAA and 0.1 mg/l BAP combined and used without or with 100 ml/l CM on RC medium.

Root explants obtained from field growing plants, aseptic seedlings and *in vitro* differentiated roots were cultured with various combinations of 2,4-D, NAA, Kn and BAP. Explants taken from seedling roots and those from *in vitro* differentiated root, cultured with 1 mg/l 2,4-D, 100 ml/l CM and 0.1 mg/l NAA combined with 0.1 mg/l BAP, both without and with 100 ml/l CM gave the best response for root callus formation. Callus

from aseptic seedling roots, as well as from *in vitro* differentiated roots, grew equally good with 1000 mg/l CH and with standard concentration of growth regulators on AM medium and 0.1 mg/l NAA combined with 0.1 mg/l BAP and 100 ml/l CM on RC medium.

Root differentiation in stem callus occurred with 0.1 mg/l NAA and 0.1 mg/l BAP combined when used with or without 100 ml/l CM, equally on RC and MS media. Percentage of lateral roots formation was 110.86 for *in vitro* developed roots, cultured with 0.1 mg/l NAA combined with 0.1 mg/l BAP and 100 ml/l CM on RC medium. The root differentiation in root callus was achieved with 1 mg/l 2,4-D in the presence of 100 ml/l CM on RC medium.

Buds from field growing plants when cultured formed roots and later on developed shoots. Root formation and shoot development in the buds was achieved on auxinless RC medium after 24 hours treatment with 3 mg/l IAA and IBA each. Rooting and shoot development was observed as unsynchronized process in cultured buds.

Shoot differentiation in stem callus was achieved with 0.8 mg/l NAA, 100 ml/l CM, 10 mg/l biotin and 250 mg/l SDDC in RC medium. The differentiated buds grew into shoots and developed roots on the same medium in 4 weeks. The plantlets grew well with 0.8 mg/l NAA, 2 mg/l BAP, 10 mg/l biotin

and 10 ml/l ENS 9extra nitrogen source). The callus formation on plantlet's roots was not inhibited with 0.0001 to 0.01 mg/l GA_3 .

The plantlets on transfer to soil responded well when watered with half strength Knop's solution supplemented with 5 drops/litre of Berthelot's solution. The plantlets required approximately 1200 lux illumination (100 W fluorescent + 200 w tungsten) for 16 hours cycled; $28^{\circ}C \pm 1$ temperature and 70-90% humidity.

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