Pak. J. Bot., 10 (2): 141–147, 1978.

SOME STUDIES ON CALLUS FORMATION IN *RAUWOLFIA SERPENTINA* BENTH.

REHANA PERVEEN AND IHSAN ILAHI

Department of Botany, University of Peshawar, Pakistan.

Abstract

Induction and growth of callus in stem and leaf segments of *Reuwolfia serpentina* was studied using knop's solution as basal modium (BM). The effect of auxins, kinetin (Kn), coconut milk (CM) and casein hydrolysate (CH) was studied on induction and growth of callus. No callus formation was observed on BM, even by the addition of 2% sucrose, in stem and leaf segments. Low concentrations of auxins had also no effect on callus formation. Callus formed at 0.1,0.5 and 1.0mg/1 of NAA; 0.5 1.0,5.0 and 10.0mg/1 of IBA; 0.1 and 0.5mg/1 of 2,4-D in stem pieces. High concentration of auxins, such as 10mg/1 of IBA was toxic for callus growth. Copious callus formed in stem pieces at 5.0mg/1 of IBA. At 1.0mg/1 of NAA roots developed from callus which had been formed on stem pieces. In leaf segments callus formed at 0.5 and 1.0mg/1 of NAA; 0.5 and 1.0mg/1 of 2,4-D. 1.0mg/1 of 2,4-D exhibited better results than 1.0mg/1 of NAA. Callus when excised from the explant and transferred to the same fresh medium, either ceased to grow or its further growth was extremely slow. The addition of either Kn, CM (10%v/v) or CH (500mg/1) enhanced callus growth but this enhancement was only temporary.

Introduction

Rauwolfia serpentina Benth., is a tropical plant of woody nature, belonging to the family Apocynaceae. The plant is of great medicinal value. The drug Rauwolfia is used for the treatment of high blood pressure and as a tranquillizer. It is derived from the roots of *R. serpentina,* which grows widely in India, Burma, Ceylon and Malaya (Woodson *et al.* 1957). Propagation of the plant by means of seeds has so far not been satisfactory. Germination of seeds of *R.serpentina* showed the range of germinability varying from 10-75% (Mitra, 1975). Vegetative propagation is possible through cuttings, but plant multiplication by this method is much too slow.

In recent years, there has been increasing interest in tissue cultures as an alternative in the asexual propagation of plants. Many valuable plants can be rapidly propagated by the use of tissue and organ culture which otherwise cannot be easily propagated by seeds or vegetative means e.g., orchids (Morel. 1966), *Gerbera* (Murashige *et al.*, 1974). *Asparagus* (Dore, 1975) and *Chrysenthemum* (Ben-Jaacov & Langhans, 1972). The procedure involves 3 steps, 1-Establishment of aseptic culture, 2- Multiplication of propagula and 3-Transfer of plantlets to soil.

Induction of callus in different parts of *R. serpentina* (Mitra & Kaul, 1964) and the growth of these calli in suitable nutrient media have been reported (Mitra *et al*, 1965). Excised roots of *R. serpentina* have been grown in continuous culture (Mitra 1968), and plantlets developed from leaf callus (Mitra & Chatturvedi, 1970).

In the present work, the effect of different growth hormones (Auxins and Kinetin), coconut Milk (CM) and casein hydrolysate (CH) was studied on callus formation in stem and leaf segments of *R. serpentina.*

Material and Methods

Stem and leaf of *R. serpentina* Benth. used for inoculation were obtained from the Medicinal plants experimental farm of the Pakistan Forest Institute, Peshawar. Knop's solution supplemented with Berthelot's solution for trace elements (Gautheret, 1939) was used as the basal medium. Two percent sucrose was used as carbon source with 0.8 % Difco-Bacto agar adjusted to pH 5.8. The medium was sterilized by autoclaving at 15 lbs/sq. inch pressure for 15 minutes.

Stem cuttings and leaves of *R. serpentina* were washed thoroughly with tap water, surface sterilized with 1% solution of $HgCl_2$ for about 10 and 7 minutes respectively, and rinsed 3 times with sterile distilled water. Stem cuttings and leaves were then cut into pieces and inoculated on the solidified agar medium in a sterile chamber equiped with u.v. light. Experiments were carried out in the dark. The cultures were kept in a cooled incubator with temperature regulated at $27 \pm 1^{\circ}C$. Indole-3-acetic acid (IAA) 0.01, 0.05, 0.5 and 1.0; Naphthalene acetic acid (NAA) 0.001, 0.005, 0.01, 0.1, 0.5 and 1.0; Indole butyric acid (IBA) 0.1, 0.5, 1.0, 5.0 and 10.0; 2,4-Dichlorophenoxy acetic acid (2,4-D), 0.1, 0.5 and 1.0; Kinetin (Kn) 0.01, 0.05, 0.1 5.0; casein hydrolysate 500 mg/l and coconut milk 10% (v/v) was used.

Results

A: Callus formation in stem pieces:

No callus formation was observed in stem segments of *R. serpentina* when inoculated on the Basal medium (BM). With the addition of 2% sucrose to the BM, swelling of stem pieces was observed and the cultures were healthier than on the BM without sucrose. In further experiments BM was always supplemented with 2% sucrose, and the effect of various auxins (NAA, IBA, 2, 4-D),Kn, CM and CH was studied on callus formation either alone or in combination (Table 1).

I. *Effect of NAA*. At low concentrations of NAA(0.001, 0.005, 0.01mg/1) callus formation was not observed. But at 0.1, 0.5 and 1mg/1 of NAA callus was formed. At 0.5 and 1.0mg/1 of NAA, callus formed earlier and exhibited comparatively a better growth than on 0.1mg/1 of NAA. Callus was white in colour, soft and friable. After 6 weeks of incubation at 1.0mg/1 of NAA, roots developed from callus induced earlier

on the stem pieces.

II. *Effect of IBA.* Callus was formed at all the concentration of IBA i.e. 0.5, 1.0, 5.0 and 10mg/1. At 0.5 and 1.0mg/1 of IBA growth of callus was very slow. However

TABLE 1. Effect of different concentrations of NAA, IBA and 2,4-D on callus formation in stem segments of *R. serpentina*.

Concentration	Auxins		
(mg/1)	NAA	IBA	2,4-D
0.001	–	–	–
0.005	–	–	–
0.01	–	–	–
0.1	+		++
0.5	++	++	+++
1.0	+++	++	
5.0		+++	
10.0		Died	

– no callus formed
\+ Some callus formed with slow growth.
++ Callus formed better than +
+++ Copious callus formed.

at 5.0mg/1 of IBA callus growth was rapid and quicker. Callus formation was observed at 10.0mg/1 but it died after some time. This concentration was found to be toxic for callus growth.

III. *Effect of 2, 4-D.* Callus formation was observed at 0.1 and 0.5 mg/l of 2, 4-D, but 0.5 mg/l exhibited better results. Callus from 0.5 mg/l was excised and transferred to either BM, BM supplemented with 0.5 mg/l of 2, 4-D in addition to either CH (500 mg/l) or CM (10 % v/v). It was observed that callus growth ceased on BM. On BM supplemented with 0.5 mg/1 of 2, 4-D further growth of callus was very slow. With the addition of CM (10 % v/v) or CH (500 mg/l) to the medium, callus growth was enhanced, but this enhancement was slight.

To study the tissue involved in callus formation, excised stem pieces were peeled off aseptically, so that outer portion having the cortex, phloem and cambium tissue was separated from the xylem and pith tissue. Both portions of the stem were inoculated on separate media. Callus formation was observed on the outer portion after one week of culture while no growth was observed on the inner portion of the stem even after 4 weeks of culture.

IV. *Effect of kinetin.* Excised stem pieces were inoculated on BM supplemented with either 0.01, 0.05, 0.1mg/1 of Kn. No callus formation was observed at these concentrations of Kn. In further experiments Kn was supplemented to the BM in combination with an auxin (NAA, IBA or 2,4-D). It was observed that Kn stimulated callus growth in combination with auxins.

V. *Effect of NAA and Kn.* Roots developed from stem pieces inoculated on BM supplemented with 1.0mg/1 of NAA. It was observed that the addition of either 0.01, 0.05 or 0.1mg/1 of Kn to the medium enhanced root development.

VI. *Effect of IBA and Kn.* Excised stem pieces were inoculated on BM supplemented with either 0.1, 0.5, 1.0, 5.0 and 10.0mg/1 of IBA and 0.01 or 0.1mg/1 of Kn. It was observed that Kn stimulated callus growth. At low concentration of IBA (0.1, 0.5, 1.0mg/1) more callus developed with the addition of Kn to the medium. The callus formed was soft, friable and white in colour. Copious callus formed at 0.5 and 5.0mg/1 of IBA with 0.1mg/1 of Kn. At 10.0mg/1 of IBA in addition to either 0.01, or 0.1mg/1 of Kn callus was formed but it died after some time.

VII. *Effect of 2,4-D and Kn.* Excised stem pieces were inoculated on BM supplemented with 0.5mg/1 of 2,4-D and 0.1mg/1 of Kn. Callus formed was white in colour, soft and friable in appearance. It was observed that callus growth was not much affected by the addition of Kn to the medium. The callus was excised and transferred to the same fresh medium. Further growth of callus was very slow. Addition of either CM (10 % v/v) or CH (500 mg/l) to the medium enhanced the callus growth but only slightly.

B. Callus formation in leaf segments:

Excised leaf segments were inoculated on BM supplemented with 2% sucrose for a duration of about 4 weeks. Callus formation was not observed at this medium. In further experiments the effects of various growth hormones (Auxins and Kn). and CH was studied on callus formation either alone or in combination (Table 2).

TABLE 2. Effect of different concentrations of IAA, NAA and 2,4-D on callus formation in leaf segments of *R. serpentina*.

Concentration (mg/1)	Auxins		
	IAA	NAA	2,4-D
0.01	–	–	
0.05	–	–	
0.1	–	–	–
0.5		+	++
1.0		++	+++

Same legends as for Table -I.

I. *Effect of IAA*:- Excised leaf pieces were inoculated on BM supplemented with 0.01, 0.05 and 0.1mg/1. No callus formation was observed.

II. *Effect of NAA* :- At low conc. of NAA i.e. 0.01 and 0.1mg/1 no callus formation occurred. However, at 0.5mg/1 of NAA swelling of the petiole and mid-rib was observed. Callus formation was noticed at 1.0mg/1 of NAA.

III. *Effect of 2,4-D* :- At 0.1mg/1 of 2,4-D, callus formation was not observed, but at 0.5 and 1.0mg/1 of 2,4-D callus developed at the cut ends of the mid-rib. Swelling of mid-rib region was also noticed at these concentrations.

IV. *Effect of Kn*:- No callus formation was observed on leaf pieces inoculated on BM supplemented with 0.01, 0.05, 0.5 and 1.0mg/1 of Kn. In further experiments Kn was supplemented to the BM in combination with auxins (IAA and NAA). It was observed that Kn had enhanced callus growth in the presence of auxins.

V. *Effect of IAA and Kn*:- At low conc. of IAA (e.g. 0.01, 0.05 and 0.1mg/1) with either 0.01, 0.05 and 0.1mg/1 of Kn some callus developed and swelling of leaf segments was observed. At 1.0mg/1 of IAA with 5.00mg/1 of Kn callus formed and roots developed from the petiole. These roots did not resume further growth on transfer to the fresh medium.

VI. *Effect of NAA and Kn*:- At low conc. of NAA (e.g. 0.1mg/1) supplemented with either 0.1 or 0.5mg/1 of Kn, no callus formation was observed. At 0.5mg/1 of NAA with 1.0mg/1 of Kn some callus developed and roots originated at the mid-rib from the upper surface of leaf but soon after their further growth ceased. These roots did not resume growth even on transfer to the fresh medium. At 1.0mg/1 of NAA 0.1mg/1 of Kn callus formation was observed.

VII. *Effect of CH*:- No callus formation occurred on leaf segemets inoculated on BM supplemented with 500 mg/l of CH. In further experiments CH was supplemented to the BM in combination with auxins (2,4-D and NAA). At 0.5 and 1.0mg/1 of NAA with 0.1 mg/1 of Kn callus formed earlier and exhibited comparatively a good growth after the addition of CH (500 mg/1) to the medium.

Discussion

From the results, it is evident that the addition of some auxin to the BM was essential to promote callus formation.

NAA at 0.1, 0.5 and 1.0 mg/l induced callus in stem segments. Callus resulted at 0.5, 1.0, 5.0 and 10.0mg/1 of IBA. IBA at 10.0mg/1 was toxic for callus growth and the callus died after some time. At 5.0mg/1 of IBA copious callus formed. 2,4-D exhibited still better results (Table I). As reported by Mitra & Kaul (1964) these studies showed that 2,4-D is more effective in callus induction in stem pieces than NAA or IBA.

In the culture of leaf segments also low conc. of auxins used had no effect on callus formation. However, 1.0mg/l of 2,4-D exhibited better callus induction than 1.0mg/l of NAA (Table 2). These findings are in conformation with those of Mitra & Kaul (1964).

Das *et al.* (1956) found that culture of pith cells of tobacco showed marked cell division in the presence of IAA and Kn. IAA or Kn alone could not produce such an effect. Similar results have been reported in tissue culture studies of *Gerbera* (Murashige *et al.* 1974) and tobacco (Murashige & Skoog, 1962). In the present work Kn alone had no effect on callus formation in stem and leaf segments. However in combination with auxins it stimulated callus growth.

Mitra & Kaul (1964) have reported the development of roots from stem pieces on a medium supplemented with IAA and adenine sulphate. In the present work it was observed, that NAA at 1.0mg/l was also effective in root initiation from callus induced on stem segments.

Mitra *et al.* (1965) noticed that the cortex, pith and phloem tissue including cambial cells enlarge and divide to form callus in stem segments. In excised leaves callus formation was confined to the vascular regions of the lamina, particularly to the mid-vein and cell division in the mesophyll tissue was not noticed. In the present work callus formation was not observed on the inner portion of the stem having xylem and pith tissue. In this case callus inducing substances might have not been able to penetrate to the pith cells, because they were inside the xylem tissue. Possibly callus might have developed if the inner core was split to expose the pith tissue.

Callus when excised from the explant and transferred to the same fresh medium either ceased to grow or its further growth was extremely slow. This showed that the explant might have been providing some substances for callus growth which was lacking in the medium. Addition of either CM (10 % v/v) or CH (50 mg/l) to the medium enhanced callus growth, but this enhancement was only slight and temporary. It seemed that salt concentration of Knop's medium was not sufficient for the continuous callus growth of *R. serpentina.* Therefore, the callus was transferred to the Murashige & Skoog (MS) medium (the significant characteristic of MS medium is its high conc., of ammonium and potassium salts) containing vitamins, sucrose and growth hormones. Better callus growth was noticed on this medium. Further work is in progress which will be reported in a later communication.

Acknowledgements

Thanks are due to Drs. M.A.F. Faridi, B.A. Siddiqi, S.Khan for help and cooperation. This project was supported by a research grant to one of us (I.I) by the University Grants Commission.

References

Ben, Jaccov, J. and R.W. Langhans. 1972. Rapid multiplication of *Chrysenthemum* plants by stem tip proliferation. Hort. Science, 7: 289–290.

Das, N.K., K. Patua and F. Skoog. 1956. Initiation of mitosis and cell division by kinetin and Indole acetic acid in excised tobacco pith tissue. Physiol. Plant., 2: 460 - 651.

Dore, Claire. 1975. Clonal multiplication of *Asparagus* through *in vitro* culture. Its use in breeding. Ann. melior plant., 25: 201 – 224.

Gautheret, J.R. 1939. Sur less possibilities de realiser la culture indefinie des tissue de tubercules de carotte. Compt. Rend. Acad. Sci., 252: 3864.

Mitra, G.C. 1968. *In vitro* growth of excised roots of *R. serpentina* in continuous culture. Indian J. Exp. Biol., 6: 230 – 231.

Mitra, G.C. 1975 a. Studies on the growth of excised roots of *R. serpentina.* Effects of certain auxins, antiauxins, amino acid, and kinetin. In "Form, structure and function in plants" (eds. H, Y. Mohan, J.H. Shah and C.K. Shah). P. 230 - 235. Sarita Prakashan, Neerat, India.

Mitra, G.C. 1975 b. Studies on the formation of viable and non-viable seeds in *R. serpentina* Benth. Indian J.Exp. Biol., 14: 54 - 56.

Mitra, G.C., C. Prabha and H.C. Chatturvedi, 1965. Histogenesis of callus tissue from different organs of *R. serpentina* Benth. In tissue culture. Indian J.Exp. Biol., 3: 216 - 22.

Mitra, G.C., H.C. Chatturvedi. 1970. Fruiting plants from *in vitro* grown leaf tissue of *R. serpentina* Benth. Curr. Sci., 39: 128-129.

Morel, G. 1966. Clonal propagation of orchids by meristem culture. Cymbidium Soc. News., 20: 3 - 11.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant., 15: 437 - 497.

Murashige, T., M. Serpa and J.B. Jones, 1974. Clonal multiplication of *Gerbera* through tissue culture. Hort. Sci., 2: 170 - 180.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultured *in vitro.* In Soc. Exp. Biol. Symp., 11: 118 – 131.

Woodson, R.E., H.W. Youngken, E. Schlitter and J.A. Schneider. 1957. "Rauwolfia. Botany, Pharmacognosy, Chemistry and Pharmacology". Little Brown and Company, Boston.