Plant Cell Reports (1994) 13:619–622

Plant Cell Reports

Organic nitrogen stimulates caulogenesis from hypocotyl callus of *Phyllanthus fraternus*

S. Rajasubramaniam and P. Pardha Saradhi

Centre for Biosciences, Jamia Millia Islamia, New Delhi-110 025, India

Received 28 September 1993/Revised version received 8 April 1994 – Communicated by G. C. Phillips

Abstract. Factors involved in promoting caulogenesis from hypocotyl explants of *Phyllanthus fraternus* were studied. Hypocotyl explants were cultured on B_5 medium supplemented with 2,4-D or NAA in the presence and absence of BAP (at concentrations 0, 10^{-7}, 10^{-6} and 10^{-5}M). Adventitious shoots differentiated from callus developed from the cut ends of 12.5% of the hypocotyl segments cultured on medium supplemented with 10^{-6}M BAP in combination with 10^{-7}M 2,4-D or 10^{-6}M NAA. Profuse rooting occurred from the hypocotyl explants on medium supplemented with 10^{-6}M BAP + 10^{-6}M NAA. Incorporation of casein hydrolysate in B_5 medium along with 10^{-6}M BAP + 10^{-7}M 2,4-D enhanced the frequency of cultures with adventitious shoots up to 68.0%. Glutamine, glutamic acid or proline could partially substitute for the effect of casein hydrolysate. Amongst the hypocotyls from 3-14 d old seedlings, the best caulogenesis was obtained with hypocotyls from 7 d old seedlings both in presence or absence of casein hydrolysate. Best rooting of shoots was achieved on half-strength B_5 medium supplemented with 10^{-6}M IBA. After hardening, plantlets were successfully transferred to the soil.

Abbreviation BAP: 6-benzylaminopurine; 2,4-D: 2, 4-dichlorophenoxyacetic acid; IBA: indole-3-butyric acid ; NAA: 1-naphthaleneacetic acid; CH: casein hydrolysate; Arg: L-arginine; Glu: L-glutamic acid; Gln: L-glutamine; Leu: L-leucine; Lys: L-lysine; Pro: L-proline.

Introduction

Phyllanthus fraternus (Euphorbiaceae) is an underexploited herbaceous medicinal plant used in traditional and folk medicine. An extract from these plants has been shown to possess anti-viral properties (especially against Hepatitis viruses) (Blumberg et al., 1989; Thyagarajan et al., 1988 ; Unander, 1991) . Extracts of *P.fraternus* have also been shown to have stomachic, diuretic and antipyretic properties (Chopra et al., 1956)

Members of Euphorbiaceae, viz. *Euphorbia esula* and *E. lathyris*, produce adventitious shoots from hypocotyls of intact seedlings grown under natural conditions (Preece and Ripley, 1992). Nataraja et al. (1973) reported the formation of shoot buds from cortical cells of hypocotyls of intact in vitro grown seedlings of *E. pulcherrima*. The present investigations were carried out with the aim of inducing caulogenesis from hypocotyl-derived callus of *P. fraternus* followed by regeneration of plants.

Material and methods

Seeds of *P. fraternus* were collected from Jamia Millia Islamia campus. Seeds were treated with ~0.5% Teepol B-300 (National Organic Chemical Industries Ltd., Bombay) for 2 min and washed with distilled water. They were then surface disinfected with 0.1% $HgCl_2$ for 3 min and washed thoroughly with sterile distilled water. The disinfected seeds were placed on 0.8% agar (RM 026 Himedia Laboratories Private Limited, Bombay) in culture tubes (150 x 25 mm containing 20 ml per tube) to raise sterile seedlings. The cultures were maintained at 25±2°C with a light/dark cycle of 14 h/10 h. White, cool fluorescent light of photon flux ~100 umol m^{-2} s^{-1} (Philips, India) was used. Hypocotyl segments (8-10 mm long) were excised (~2 mm below the cotyledonary node) from 7 d old seedlings and were cultured on modified B_5 medium (Neena and Pardha Saradhi, 1992) gelled with 0.8% agar. The medium was supplemented with 2,4-D, NAA or BAP alone or in various combinations (at concentrations 0, 10^{-7}, 10^{-6} and 10^{-5}M). The pH of the growth medium was adjusted to 5.8 and was autoclaved at 1.06 kg cm^{-2} for 15 min. In order to test the effect of organic nitrogen on caulogenesis, B_5 medium containing 10^{-6}M BAP + 10^{-7}M 2,4-D was further supplemented with casein hydrolysate (at 0, 100, 200 or 500 mg/l), L-arginine, L-glutamic acid, L-glutamine, L- glycine, L-leucine, L-lysine or L-proline (at concentrations 0, 0.1, 1, 5 or 10 mM).

To check whether the age of the hypocotyl donor seedlings affect shoot inducing ability, hypocotyl explants from 3-14 d old seedlings were grown on B_5 medium supplemented with 10^{-6}M BAP + 10^{-7}M 2,4-D both in the presence and absence of 200 mg/l CH.

For rooting, shoots (~2 cm long) were transferred onto Whatman No. 1 filter paper bridges in culture tubes containing 15 ml of

Correspondence to: P. Pardha Saradhi

half-strength B_5 liquid medium supplemented with IBA or NAA at concentrations 0, 10^{-7}, 10^{-6} or 10^{-5}M. Rooted shoots were then hardened by transferring them to half-strength B_5 medium lacking sucrose and other organics. These plantlets were transferred to pots (90 x 110 mm) containing autoclaved mixture of perlite, loamy soil and coarse sand (1:1:2 by vol), covered with thin perforated transparent polyethylene bags and were kept in a growth chamber (maintained at 25°C and with light conditions similar to that mentioned earlier) for 7 d for acclimatization. These plantlets were irrigated initially with one-fourth strength and subsequently with one-tenth strength B_5 medium lacking organics. Polyethylene bags were removed from the pots on day 14 and the potted plantlets were finally transferred to the garden on day 18.

At least 24 replicates were used for every treatment and all experiments were conducted at least 3 times.

Results and Discussion

Hypocotyl explants from 7 d old *P. fraternus* seedlings, cultured on B_5 medium supplemented with 2,4-D or NAA, initiated callus from the cut ends 7 d after culture, followed by proliferation of callus from all over the surface. Best callus induction and proliferation occurred on medium with 10^{-7}M 2,4-D (Fig. 1A). Occasional root induction was observed in some of the cultures grown on this medium. The roots induced in these cultures were hyaloid and swollen. Callus inducing ability of 2,4-D decreased with an increase in its concentration. In contrast, callus inducing ability of NAA increased with increase in concentration (Table 1). Presence of NAA in medium also resulted in differentiation of roots from callus (Fig. 1B), and the number of roots from callus increased with the increasing concentrations of NAA (Table 1).

Table 1. Effect of 2,4-D and NAA on callus and root formation from hypocotyl explants of *P. fraternus* grown on B_5 medium.

Auxins in M	% cultures callusing	Relative callus growth	% cultures rooting	No. of roots per culture
2,4-D				
0	-	-	10.4	1
10^{-7}	100.0	+++	6.2	3-4
10^{-6}	83.3	++	-	-
10^{-5}	83.3	+	-	-
NAA				
0	-	-	10.4	1
10^{-7}	83.3	+	100.0	7-8
10^{-6}	83.3	++	100.0	10-15
10^{-5}	100.0	+++	100.0	35-40

Mean of 3 independent experiments each with 24 replicates. Data recorded at the end of 28 d culture.

Unander (1991) reported induction of callus from stem and branch pieces of *Phyllanthus amarus*, *P. urinaria* and *P. abnormis* in presence of 2,4-D or IBA alone and in combination with BAP. Further, he observed optimal callus induction and growth when either of the auxins was used along with BAP. In contrast, during the present studies, optimal induction and growth of the callus was observed from hypocotyl segments of *P. fraternus* when 2,4-D was used alone. But the presence of BAP along with either of the auxins (2,4-D or NAA) resulted in compact callus formation. Callus formed on B_5 medium supplemented with 10^{-6}M BAP + 10^{-7}M 2,4-D (hereafter referred to as BMM), developed 1 or 2 minute dark green patches 15 d after inoculation. Subsequently, 5-6 d later, shoots originated from such patches in 12.5% cultures (Fig. 2). Shoot buds were also induced from ~12.5% cultures on medium supplemented with 10^{-6}M BAP + 10^{-6}M NAA. All the cultures grown in the latter medium showed profuse rooting.

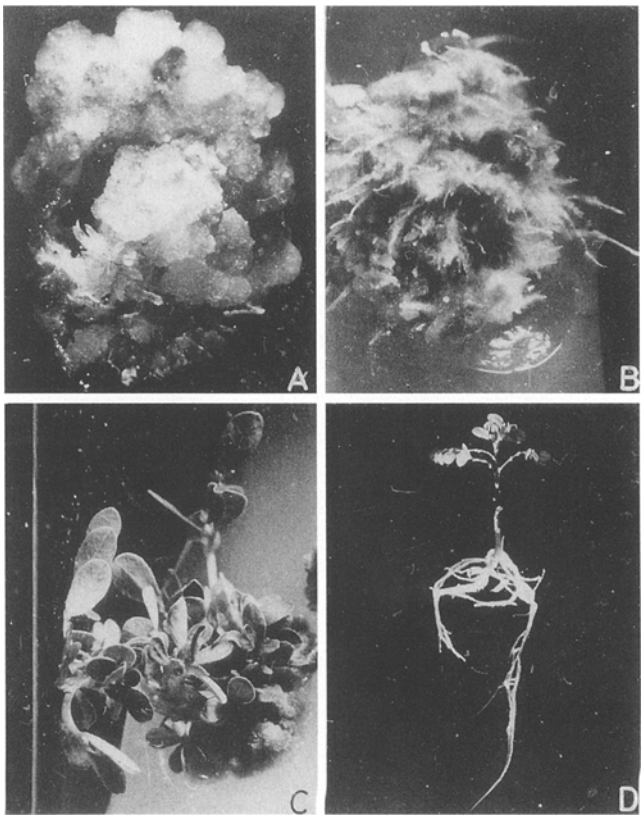

Fig.1 A. Hypocotyl culture of *Phyllanthus fraternus* raised on B_5 medium supplemented with 2,4-D 10^{-7}M showing callusing after 30 d. (x 2)

B. Hypocotyl culture of *P. fraternus* showing rhizogenesis on B_5 medium supplemented with NAA 10^{-7}M after 30 d. (x 1.8)

C. Hypocotyl culture of *P. fraternus* showing caulogenesis on B_5 medium supplemented with 10^{-6}M BAP + 10^{-7}M 2,4-D + CH 200 mg/l after 25 d. (x 1.8)

D. Plantlet of *P.fraternus* obtained after rooting of shoot from 'C' on half-strength B5 medium supplemented with IBA 10^{-6}M. 20 d after transfer. (x 0.95)

The quality and quantity of the nitrogen source has been reported to influence organogenesis in tissue cultures (Veliky and Dyson Rose, 1973). Natural extracts such as CH have been reported to promote callus induction and enhance morphogenesis in tissue cultures including those of certain Euphorbs (Hirabayashi et al., 1992; Liu et al., 1992; Johri and Srivastava, 1973; Srivastava, 1971, 1973; Yamamoto, 1991). In the present studies, the presence of CH (at all concentrations tested) enhanced shoot inducing ability of hypocotyl explants grown on BMM (Fig. 2A). About 5.5 fold increase in cultures showing caulogenesis was noted on BMM supplemented with 200 mg/l CH. In addition, CH also increased the number of shoots formed per culture. The number of shoots on this medium varied between 5-15 (Fig. 1C), as compared to 1 or 2 on BMM.

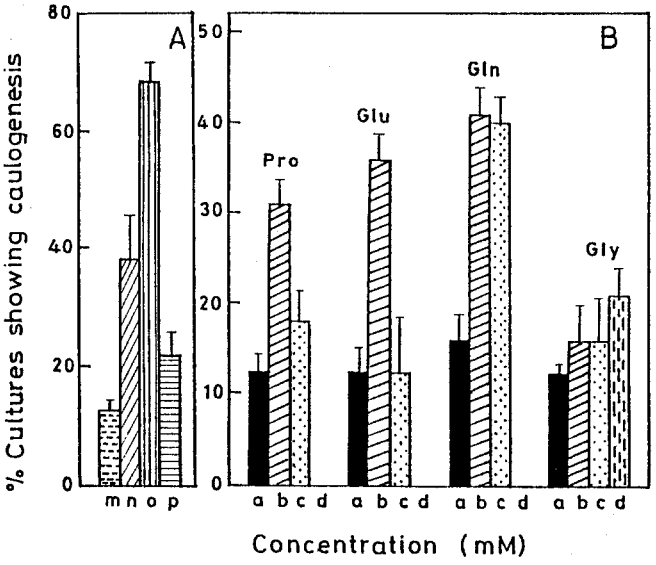

Fig 2. Effect of different concentrations of CH and amino acids on the percentage of *P. fraternus* cultures showing caulogenesis. m, n, o and p represent 0, 100, 200 and 500 mg/l of CH respectively. a, b, c, and d represent 0.1, 1, 5, and 10 mM of the amino acids respectively. The vertical lines above the bars represent standard deviation from the mean values of 3 independent experiments. Data scored after 28 d culture.

The age of explants has been reported to influence organogenesis in tissue cultures (Clog et al., 1990; Sharma et al., 1990). Response of hypocotyl explants obtained from different aged seedlings cultured on BMM and BMM supplemented with CH is depicted in Fig. 3. Irrespective of the age of the seedling from which the hypocotyl segments were obtained, the frequency of cultures exhibiting shoot induction was higher in the presence of CH than in its absence. Hypocotyl explants from 7 d old seedlings showed higher caulogenic capacity than those from younger or older seedlings (Fig. 3).

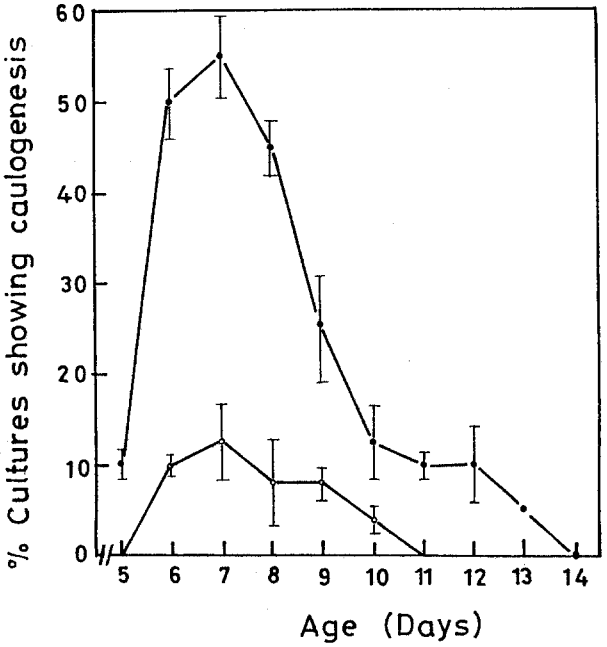

Fig 3. Frequency of caulogenesis of *P. fraternus* from hypocotyl explants of different aged seedlings on B_5 medium supplemented with 10^{-6}M BAP + 10^{-7}M 2,4-D in absence (○) and presence (●) of CH (200 mg/l). Data are the mean of 3 independent experiments ± standard deviation. Data scored after 28 d culture.

Casein hydrolysate is known to be rich in amino acids (Johri and Srivastava, 1973) and amino acids have been reported to enhance caulogenesis in cultures (Basu et al., 1989; Freytag et al., 1988; Shetty et al., 1992). Hence attempts were made to replace CH by amino acids at concentrations ranging from 0.1 to 10 mM. Among various amino acids used, Gln, Glu and Pro significantly promoted shoot induction from hypocotyl cultures grown on BMM (Fig. 4). In contrast, Leu inhibited shoot induction. Figure 2B depicts the effect of various concentrations of Gln, Glu, Gly and Pro on the frequency of cultures showing shoot induction. Gln, Glu and Pro brought about maximal shoot induction at 1 mM, with Gln and Glu showing the strongest promotive effect. No shoot induction was observed when the concentration of Gln, Glu or Pro was elevated to 10mM. It is clear from Figs. 4 and 2B that none of the amino acids used singly could completely substitute for the stimulatory effect of CH, thereby suggesting the necessity of a balance of various amino acids for optimal shoot induction. We conclude that organic nitrogen is one of the essential factors for promoting shoot induction from hypocotyl cultures in *Phyllanthus fraternus*.

Rooting of shoots

Auxins such as IBA and NAA are widely employed to induce rooting (Hussey, 1986; Wakhlu et al., 1989).

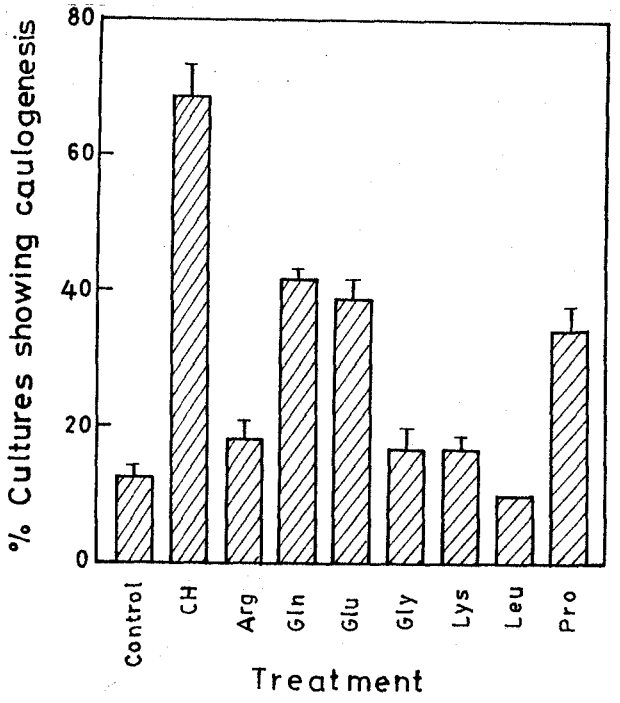

Fig 4. Effect of CH (200 mg/l) and amino acids (1 mM) on the frequency of *P. fraternus* cultures showing caulogenesis. Data scored after 28 d culture.

In the present investigations, shoots obtained on BMM + 200 mg/l CH were excised and transferred to half-strength liquid B_5 medium supplemented with IBA or NAA (at concentrations 0, 10^{-7}, 10^{-6} or 10^{-5}M). Root initiation was observed within 7-8 d from the cut end of shoots. Both IBA and NAA promoted initiation of roots at all concentrations tested. However, at 10^{-5}M they also induced callus from the cut ends. Best root induction, with both the auxins, was observed at 10^{-6}M. However, the roots obtained with IBA were healthier and longer (Fig. 1D) than with NAA. Plantlets were hardened and transferred to the garden, where 70% have survived.

Acknowledgement. Financial support to one of us (S.R.) in the form of Senior Research Fellowship from University Grants Commission is gratefully acknowledged.

References

Basu A, Sethi U, Guha-Mukherjee S (1989) Plant Cell Reports 8: 333-335

Blumberg BS, Millman, Venkateswaran PS, Thyagarajan SP (1989) Cancer Detect Prev 14: 195-201

Chopra RN, Nayar SL, Chopra IC (1956) (eds) Glossary of Indian medicinal plants. Revised edition 1986, Publications and Information Directorate, CSIR, New Delhi 110 012

Clog E, Bass P, Walter B (1990) Plant Cell Reports 8: 726-728

Freytag AH, Anand SC, Rao-Arelli AP, Owens LD (1988) Plant Cell Reports 7: 30-34

Hirabayashi, Taihei, Misoo S, Kamajima O, Sawano M (1992) Sci Rep Fac Agric KOBE Univ 20: 23-29

Hussey G (1986) In: Yeoman M M (ed) Plant cell culture technology, Botanical Monographs, Vol 23, Blackwell Scientific Publ Ltd, London, pp 29-66

Johri BM, Srivastava PS (1973) In: Murthy YS, Johri BM, Mohan Ram HY, Verghese TM (eds) Advances in Plant Morphology, Puri V Commemoration Vol, Sarita Prakashan, Meerut, India, pp . 339-358

Liu HJ, Shuji M, Kamajima O, Sawano M (1992) Jpn J Breed 42: 367-373

Nataraja K, Chennaveeraiah MS, Girigowda P (1973) Curr Sci 42: 577-578

Neena K, Pardha Saradhi P (1992) Plant Cell Reports 11: 476-479

Preece JE, Ripley KP (1992) In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, Vol 20, High-Tech and micro-propagation IV, Springer-Verlag, Berlin, pp 91-112

Sharma KK, Bhojwani SS, Thorpe TA (1990) Plant Sci 66: 247-253

Shetty K, Shetty GA, Nakazaki Y, Yoshioka K, Asano Y, Oosawa K (1992) Plant Sci 84: 193-199

Srivastava PS (1971) Z Pflanzenphysiol 66: 93-96

Srivastava PS (1973) Z Pflanzenphysiol 69: 270-273

Thyagarajan SP, Subramanaian S, Thirunalasundari T, Venkateswaran PS, Blumberg BS (1988) Lancet 2: 764-766

Unander DW (1991) Plant Cell Reports 10: 461-466

Veliky IA, Dyson Rose (1973) Can J Bot 51: 1837-1844

Wakhlu AK, Barna KS (1989) Plant Cell Tiss Org Cult 17: 235-241

Yamamoto Y (1991) In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, Vol 15, Medicinal and aromatic plants, Springer-Verlag, Berlin, pp 247-257