BIOLOGIA PLANTARUM 42 (2): 169-176, 1999

Changes in isozyme patterns during *in vitro* regeneration from cotyledon explants of *Brassica* species

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Abstract

High frequency shoot regeneration from cotyledons excised from 4-d-old seedlings of *Brassica campestris* L. cv. M 27 and *Brassica juncea* (L.) Czern. cv. Pusabold was achieved on Murashige and Skoog's (MS) medium supplemented with 1.0 mg dm^{-3} N^6-benzyladenine (BA) and 3 % (m/v) saccharose. Rooting occurred simultaneously with shoot formation on the medium containing 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} 1-naphthaleneacetic acid. Cultures of cotyledon, cotyledon derived non-differentiating calli and differentiated calli with shoots and/or roots were analysed at different intervals for isozyme patterns of esterase and peroxidase. With the BA-induced development of shoot and/or root from non-differentiating callus, some conspicuous isozymes appeared which indicates the involvement of these isozymes in root and shoot development rather than in induction of morphogenesis in callus.

Additional key words: esterase, growth regulators, PAGE, peroxidase.

Introduction

Several reports of regeneration from seedling explants of *Brassica* species have been published during the past decade (Jaiswal *et al.* 1987, George and Rao 1980, Murata and Orton 1987, Horeau *et al.* 1988, Sato *et al.* 1989, Kozai *et al.* 1991, Sharma *et al.* 1990, 1991, Burnett *et al.* 1992). Lazzeri and Dunwell (1986) and Murata and Orton (1987) reported that cotyledon explants of *Brassica oleracea* exhibit poor and sporadic differentiation, but Horeau *et al.* (1988) reported high frequency regeneration from excised cotyledons of three cultivars of this species. The

Received 5 May 1998, *accepted* 27 September 1998.

Abbreviations: BA - N^6-benzyladenine; Kn - kinetin; NAA - 1-napthaleneacetic acid; MS - Murashige and Skoog's medium; Zn - zeatin.

Acknowledgements: The authors wish to acknowledge the Department Forests & Environment, Government of Orissa for providing necessary facilities.

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variability in shoot formation from cotyledons of several *Brassica* species is attributable to experimental conditions (Murata and Orton 1987). Till date, there is no report showing association between isozyme patterns and *in vitro* plant regeneration from seedling explants of these species. Therefore, in the present investigation we determined esterase and peroxidase isozyme patterns during various stages of plantlet differentiation from cotyledon-derived-explants of *Brassica campestris* and *B. juncea*.

Materials and methods

Plants and *in vitro* cultivation: Seeds of *Brassica campestris* L. cv. M27 and *B. juncea* (L.) Czern. cv. Pusabold were collected from Orissa University of Agriculture and Technology, Bhubaneswar and surface sterilized with 0.1 % mercuric chloride (m/v) solution for 10 min, followed by 3 rinses in sterile distilled water. The seeds (8 - 10) were placed on germination medium comprising of Murashige and Skoog (1962) (MS) salts and vitamins, 3 % (m/v) saccharose and 0.8 % (m/v) *Difco-Bacto* agar. The culture tubes (25 × 150 mm) were incubated at 25 ± 2 °C under cool white fluorescent lamps (*Philips*, Bombay, India), a photon flux density of 55 μmol m^{-2} s^{-1} at the culture surface for a 16-h photoperiod.

Individual cotyledons were excised from 4-d-old seedlings (or from 5 and 8-d-old seedlings in one experiment) about 1 - 2 mm below the cotyledonary node. The cotyledon explants measured 5 - 6 mm and contained a bilobed lamina and a short (about 2 mm long) petiole. The cotyledons were planted with their abaxial surface in contact with the medium and the proximal cut end embedded in the medium. All the experiments were conducted using half-strength MS medium supplemented with BA, kinetin (Kn), zeatin (Zn) and NAA in concentration 0.5 - 2.0 mg dm^{-3}. Usually, 20 cultures were used per treatment and the experiment was performed three times. The morphological changes were recorded on the basis of visual observations.

Enzyme extraction and detection: Each sample (0.5 g) of cotyledon collected from 4-d-old seedlings (early phase), non-differentiating callus after 15-d of culture (mid-term phase) and callus showing shoots and/or roots differentiation after 4 weeks of culture (late phase) were homogenised with 0.2 M Tris-HCl and adjusted to pH 8.5, containing 1 M saccharose and 0.056 M 2-mercaptoethanol (Wetter and Dyck 1983). The crude homogenates were then centrifuged at 12 000 *g* for 30 min to remove cellular debris. The supernatant was either used directly for electrophoresis or stored at a temperature of -25 °C. All the extractions were made at a temperature of approximately 4 °C.

Isozymes were separated into discrete bands by tube polyacrylamide gel electrophoresis (PAGE) using a stacking gel density of 0.6 % (m/v) N,N^1-methylene bisacrylamide and 2.5 % (m/v) acrylamide and a resolving gel of 0.2 % (m/v) and 7.5 % (m/v), respectively, of these compounds and a running buffer of 0.005 M Tris-glycine, pH 8.3. Gels were precooled to 2 - 5 °C prior to each electrophoresis. Extracts were prepared by the addition of 0.005 cm^3 bromophenol blue (BPB) (0.05%, m/v) and 0.050 cm^3 of this extract added to each tube. Electrophoresis was

performed in the dark at 5 °C using 4 mA/tube for 90 - 120 min. Immediately, after each electrophoretic run, gels were stained for esterase (EST) activity at room temperature using 1.31 mM α-naphthylacetate, 0.17 mM acetone and 2.79 mM Fast Blue RR salt in 0.2 M phosphate buffer, pH 6.0 (Wetter and Dyck 1983) and for peroxidase (PXR) activity using 2 mM *o*-dianisidine, 2.01 mM β-naphthol in 0.1 M Tris-acetate buffer, pH 4.0, 3.44 mM acetone, 0.029 mM 30 % H_2O_2 and 100 cm^3 distilled water (Eduardo 1983). After staining, the gels were photographed, diagrams made and stored in 7 % (v/v) acetic acid. The position of the isozyme band in the gel was expressed as relative mobility (R_f) by measuring the distance migrated by the particular band relative to that of bromophenol blue used as tracking dye during electrophoresis.

Results and discussion

Our results imply that the age of explants is one of the important factors in the rapid shoot bud formation in cotyledonary callus of *Brassica* species. For cotyledon explants taken from 1- to 8-d-old seedlings and cultured on MS medium supplemented with 1.0 mg dm^{-3} BA, the percentage of explants with shoot bud differentiation increases with age of seedlings, reaching a maximum at 4 d (Fig. 1). Thereafter, the response sharply declines and the cotyledons from 8-d-old seedlings show negligible regeneration as reported earlier (Sharma *et al.* 1990, Hachey *et al.* 1991).

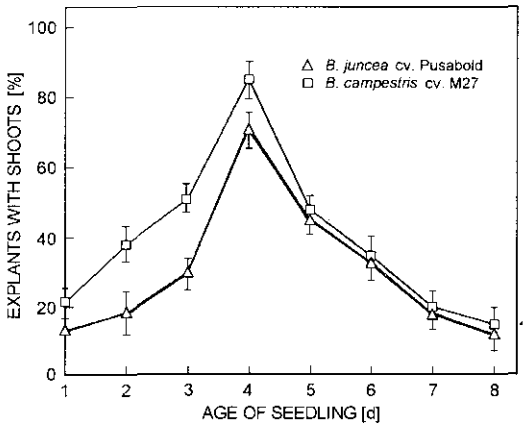

Fig. 1. Regeneration of shoot buds from cotyledon explants of *B. campestris* cv. M27 and *B. juncea* cv. Pusabold obtained from donor seedlings of different age (MS medium + 1.0 mg dm^{-3} BA). Means ± SE from 20 replicates.

Among the three cytokinins tried at various concentrations (0.5, 1.0, 1.5 and 2.0 mg dm^{-3}), BA was the most effective in terms of the number of explants forming shoots and the number of shoots per explant (data not shown). Although both Kn and Zn induced shoot bud regeneration at concentrations 1.0 - 1.5 mg dm^{-3}, the number of shoot buds per explant (data not shown) were reduced and poor growth occurred in

zeatin in comparison to BA. BA at a concentration of 1.0 mg dm^{-3} evokes optimal regeneration potential and subsequently shoot elongation in both *B. campestris* and *B. juncea* (Fig. 2). At higher concentration BA was also effective for inducing shoot bud regeneration, but the shoots arising on this medium were often more vitreous than those arising from explants on 1.0 mg dm^{-3} BA. Sharma *et al.* (1990) reported that cytokinin alone were sufficient for shoot bud regeneration and subsequent

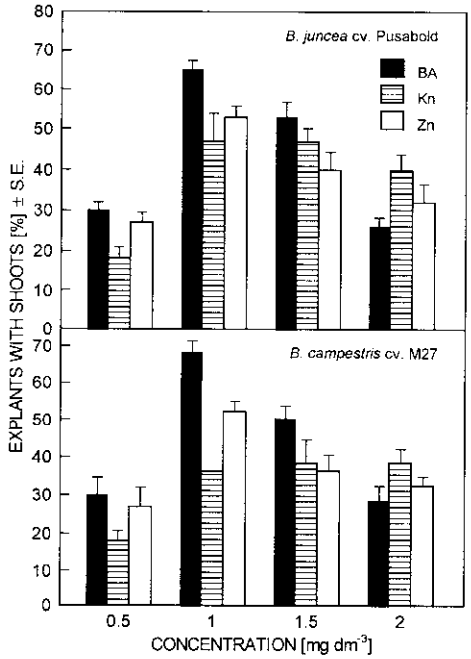

Fig. 2. Effect of BA, Kn and Zn on shoot bud differentiation in excised cotyledons of *B. campestis* and *B. juncea* after 30-d of culture on MS basal medium. Means ± SE from 20 replicates.

growth of shoots in cotyledon cultures of *B. juncea*. With the inclusion of NAA in the medium (Fig. 3), the regeneration of shoot buds is reduced but root initiation occurred as was reported earlier in *Brassica juncea* cv. Rai-5 (George and Rao 1980) and in *Raphanus sativus* (Hagazi and Matsubara 1992). The maximum number of shoots in *B. juncea* (20 per explant) and in *B. campestris* (28 per explant) is achieved in medium containing 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA. Increasing the concentration of NAA from 0.5 to 2.0 mg dm^{-3} (Fig. 3) caused decline in the production of shoots relative to the production of roots. On the contrary, an increase in the concentration of BA from 0.5 to 2.0 mg dm^{-3}caused an increase in shoot production relative to root production as reported earlier in *B. carinata* (Jaiswal *et al.* 1987), *B. napus* (Moloney *et al.* 1989) and *B. juncea* (Sharma *et al.* 1990). On the contrary, Hachey *et al.* (1991) reported that the addition of NAA in conjuction with BA reduces the rooting response and promotes caulogenic formation and regeneration.

Enzymes which are known as metabolic markers, change during development and differentiation (Chawla 1989). Based on the isozyme analysis at three developmental stages namely the 4-d-old excised cotyledon, undifferentiated callus (15-d-old) and differentiated 30-d-old shoots/roots, it has been observed that induction of morphogenesis is accompanied with the synthesis of certain proteins and enzymes. In the cotyledonary phases, EST activity was low in *B. juncea* and weak intensity band

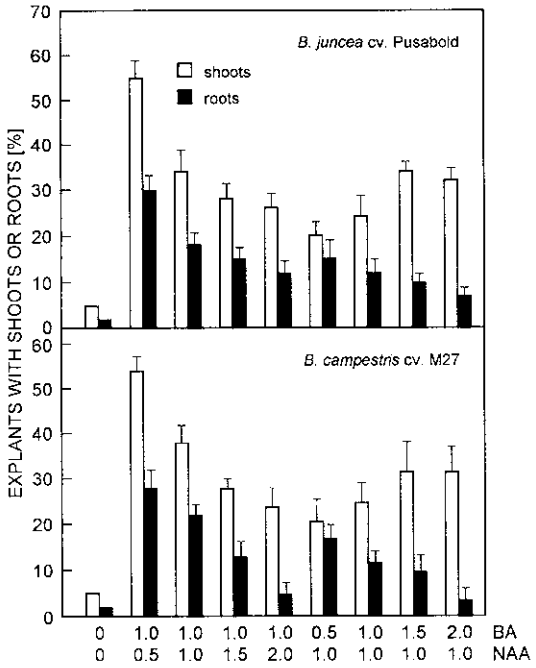

Fig. 3. Effect of NAA and BA on shoot and root formation from cotyledon explants of *Brassica* species after 30-d of culture on MS basal medium. Means ± SE from 20 replicates.

($R_f = 0.44$) appeared in *B. campestris* (Fig. 4). Similarly, for the undifferentiated callus phase, the EST isozyme profile differed in both the species. In *B. juncea* three EST bands resolved during undifferentiated callus phase (Fig. 4), having R_f values of 0.44, 0.64 and 0.72 respectively. Upon differentiation into shoots/roots phase, the dark intensity thick band disappeared and two anodal thin dark bands reappeared. In *B. campestris* three EST isoenzymes are resolved (Fig. 4) having R_f values of 0.47, 0.52 and 0.62, respectively, during undifferentiated callus phase and an additional isoenzyme appeared during the differentiation phase having an R_f value of 0.74. This might be induced during differentiation and might provide a genetic marker for differentiation (Feirer and Simon 1991). EST thus appeared inactive during the early callus phase and probably was latter triggered through genetic responses induced by certain growth regulators (Everett *et al.* 1985).

Peroxidase (PXR) activity is also modulated during developmental phases of the above two *Brassica* species (Fig. 4). During the early phase, very weak intensity

bands of PXR activity appeared in *B. juncea* (R_f = 0.47). In the differentiated shoots/roots phase of *B. juncea*, the peroxidase activity was pronounced showing thick dark cathodal band having Rf value ranging from 0.02 to 0.58 (Fig. 4). In

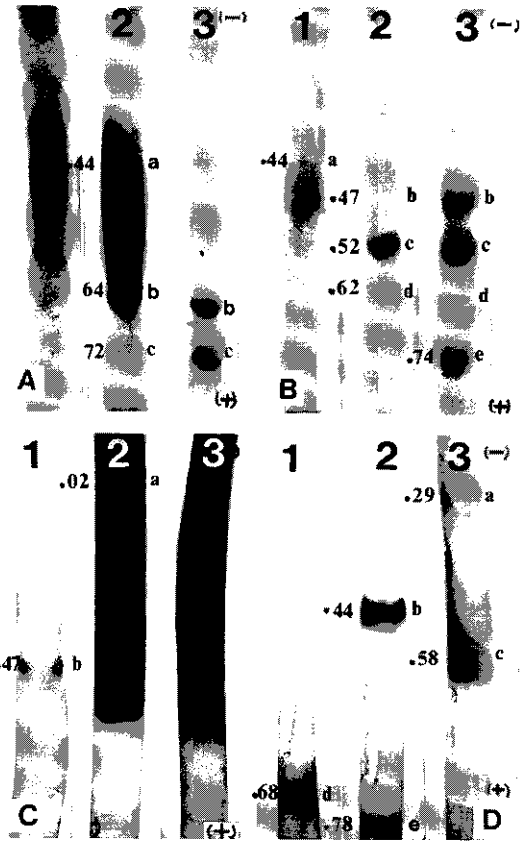

Fig. 4. PAGE zymograms of esterase (*A,B*) and peroxidase (*C,D*) isozyme patterns of *B juncea* cv. Pusabold (*A,C*) and *B. campestris* cv. M27 (*B,D*) cultured on MS medium + 1.0 mg dm^{-3} BA + 0.5 mg dm^{-3} NAA. Samples were obtained from cotyledon tissue of 4-d seedling (1), undifferentiated callus derived from cotyledon after 15-d of culture (2) and differentiated callus with shoots/roots after 30-d of culture (3). Equal amount of enzyme extracts were loaded onto each tube (0.050 cm^3).

B. campestris, the peroxidase activity was very weak at the undifferentiated callus phase with one cathodal band (R_f = 0.44) and one anodal band (R_f = 0.78). Upon differentiation into shoots/roots phase, two new thick dark bands (R_f = 0.29, 0.58) appeared and the anodal band (R_f = 0.78) disappeared. This result confirmed earlier reports (Del Grosso and Alicchio 1981, Gaspar *et al.* 1985, Berthon *et al.* 1987).

In conclusion, the isoenzyme activity was low during early phase and increased progressively during late phases. Multiple isoenzymes have been found in

undifferentiated callus phase as well as in differentiated shoots/roots phase. Isozymic patterns might provide information useful in characterization of other *Brassica* species and their wild relatives.

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