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Regeneration Potentiality and Isozymic Variations During Morphogenesis of Barley Callus

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Abstract. Morphogenic callus cultures were obtained from 7–10 days old immature embryo explants on Murashige and Skoog and Gamborg's medium supplemented with 2,4-D. In the initial stages of culture the frequency of shoot formation varied from 28% to 65%. After 5 to 6 months of subculturing, the frequency of shoot formation was reduced to 14%. In the initial stages of culture, growth hormones do not seem to be very important for regeneration. Cultures from young and old non-differentiating calli, and calli with shoot and/or root formation at different intervals were analysed for isozymes of esterase, peroxidase and acid phosphatase for studying the morphogenic capacity. With the development of shoot/root, changes in isozymes takes place but no specific isozyme(s) could be related to the process of induction of morphogenesis.

Additional index words: Hordeum vulgare, plant regeneration, esterase, peroxidase, acid phosphatase

The development of cell and tissue culture methods in crop plants has recently received considerable attention. These *in vitro* techniques offer a system for rapid propagation of a superior genotype and selection at the cell or callus level against phytotoxins (Chawla and Wenzel 1987a). It also provides a means of studying the fundamental life processes through the study of enzymes, which commonly exist in multiple molecular forms (isozymes) some of which are only expressed at particular stage of development and differentiation (Scandalios 1974). The isozyme profile of a tissue is thus a reflection of the state of differentiation of its component cells. Although isozymes have been recommended as sensitive phenotypic and genotypic markers in plant tissue cultures, there are few examples of their use in studying the differentiation of cultured plant cells (Scandalios and Sörenson 1977, Marselak 1981, Prasanna *et al.* 1983). Everett *et al.* (1985) studied esterase and glutamate dehydrogenase in embryogenic and shoot forming cultures of maize. This paper describes the regeneration potentiality of callus derived from embryos and the changes in different enzymes patterns when callus shows differentiation into shoots and/or roots.

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MATERIALS AND METHODS

Two cultivars of barley (*Hordeum vulgare* L. cv. Dissa and cv. Igri) and two F_2 families "W 193" and "W 195" were grown in the greenhouse under semi-controlled conditions. Callus cultures from immature embryos were initiated on Murashige and Skoog (1962) (MS) medium and B5 medium of Gamborg *et al.* (1968) when the spikes were 7–10 d old as described by Chawla and Wenzel (1987b). Both the media were supplemented with 2 mg l^{-1} 2,4-D. Callus was regularly sub-cultured at an interval of 4–5 weeks. After culturing at least once on the initiation media, as many parts as possible of the calli were placed on different B5 media supplemented with different phytohormone concentrations and combinations including CH and kept under 16 h photoperiod (*ca* 2000 lx).

For enzymic studies, the hard, compact and nodular calli of Dissa and Igri were put on regeneration media containing 0.35 mg l^{-1} NAA and 1 mg l^{-1} BAP. Samples from ND-Y – young non-differentiating callus, ND-0 – about one year old non-differentiating callus, and CS – callus showing shoots and CR – callus with roots after 3 weeks of growth in regeneration medium were taken. These were sampled again after 5 weeks of growth in regeneration medium for CS – callus with shoots and C – callus and S – shoots separately.

Polyacrylamide gel electrophoresis: Samples of callus or shoots material (0.5 g) was homogenised in 0.8 ml Tris – HCl buffer (pH 6.7) containing 10% glycerol. Homogenate was centrifuged at 12 000 *g* for 20 min. A system with 7.5% (m/v) gel with discontinuous buffer system using Tris-glycine buffer (pH 8.7) was employed. The electrophoresis was carried out at 4 °C with 50–60 mA/gel.

Staining of gels: Esterases were localised by the method of Bergmann and Mann (1973), and peroxidases and acid phosphatases by the procedures given by Scandalios (1969).

RESULTS

Morphogenic callus tissue was initiated on both B5 and MS medium but the frequency of induction of calli was better on B5 medium. The scutellar callus was divided into small pieces and propagated at least once on the same medium before the vigorous calli were transferred to different regeneration media. In the initial stages of culture were not much differences in the maximum regeneration potentiality when the medium was supplemented with a particular combination of growth hormones or without growth hormones for a particular genotype (Table 1). The maximum frequency of regeneration varied from 49%

Abbreviations: 2,4-D – dichlorophenoxyacetic acid, NAA – naphthylacetic acid, BAP – 6-benzylaminopurine, IAA – indolyl-3-acetic acid, CH – casein hydrolysate.

TABLE 1
Regeneration frequency of calli derived from immature embryos in response to different growth substances at different stages of subcultures

Genotype	No hormone	B5 medium with growth hormones [mg l^{-1}]				
		IAA (1) zeatin (1)	IAA (1) BAP (1)	NAA (0.35) BAP (1)	NAA (1) BAP (1)	CH (200)
After one subculture						
Dissa Calli	47	40	17	18	22	19
Reg. [%]	49	38	47	33	27	37
Plants [%]	28	15	24	27	14	26
W 193 Calli	20	16	15	18	14	10
Reg. [%]	80	75	80	67	71	80
Plants [%]	65	63	54	61	42	60
W 195 Calli	18	18	–	8	8	12
Reg. [%]	56	50	–	50	13	33
Plants [%]	39	33	–	40	13	25
After 4–5 subcultures						
Dissa Calli	26	23	–	29	22	21
Reg. [%]	12	22	–	24	23	29
Plants [%]	4	9	–	14	14	10

to 80% but the maximum shoot formation ranged from 28% to 65%. Up to 8 green plants were obtained per original immature embryo with an average of 5. It has been observed that phytohormones were not essential for the regeneration process from young cultures, but low auxin and cytokinin concentrations were some times beneficial for plant formation. The best hormone combination considering all the genotypes was 0.35 mg l^{-1} NAA with 1 mg l^{-1} BAP.

The regeneration potentiality of the calli after 4 to 5 subcultures was studied in Dissa genotype. Out of 23% regenerating calli, a maximum of 14% could be regenerated into plants on the hormone combination of NAA and BAP.

Isozymic studies

Esterase: It could be resoluted into 4 activity zones (Fig. 1a). Isozymes of α, β and γ zones were present in all the stages studied. Zone δ had 7 bands. δ_3 and δ_4 isozymes appeared with the induction of morphogenesis of callus into roots or shoots. These isozymes were also present in the shoots while callus (C) separated from the shoots did not show these bands. δ_1 and δ_2 isozymes were absent in old non-differentiating callus. Both the genotypes showed the same pattern for their respective stages.

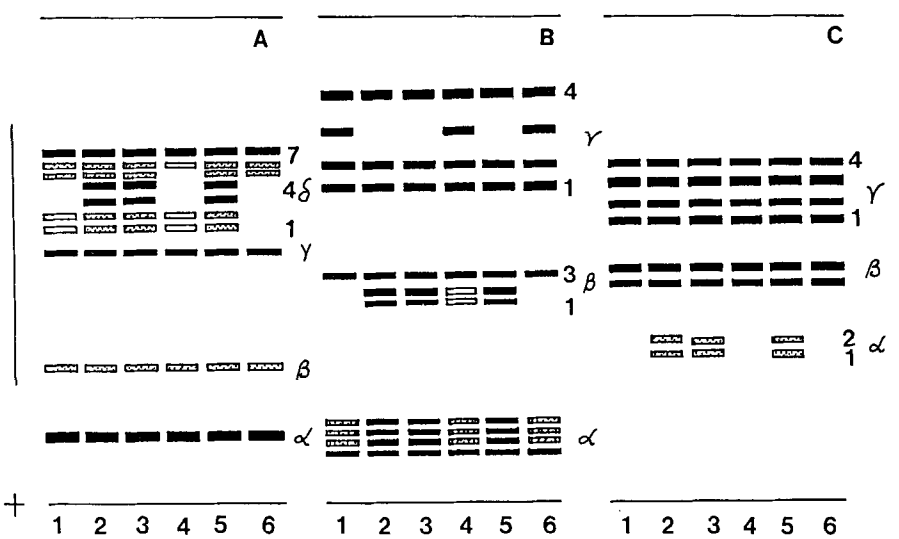

Fig. 1. Zymogram showing isozymes in barley: (A) esterase, (B) peroxidase, (C) acid phosphatase. In vertical columns: 1 – non differentiating young callus (ND-Y); 2 – callus with shoots (CS); 3 – callus with roots (CR); 4 – callus only (C); 5 – shoots only (S); 6 – non differentiating old callus (ND-0). In figures: empty column – low intensity; dotted column – medium intensity; full column – high intensity.

Peroxidase: Three main activity zones were observed. Zone α consisting of 4 bands was present in all the stages but the activity was low in the callus separated from shoot (C) and in the young and old non-differentiating callus (Fig. 1b). With the induction of morphogenesis of callus into shoots or roots, bands 1 and 2 of β zone appeared. These were present in the separated callus and shoot portions also. Band 3 in zone γ was present only in the callus portion rather than in the shoot portion. Isozymic pattern was identical in both the genotypes.

Acid phosphatase: The 8 isozymes were present in 3 zones (Fig. 1c). Bands 1 and 2 (sometimes could be clearly separated) of zone α were present in the shoot portion of tissue and in CS and CR. The same pattern was exhibited by both the genotypes for their respective stages.

DISCUSSION

Morphogenic callus tissue initiated from immature embryos gave rise to a large number of green shoots but under appropriate conditions of media, embryo size, temperature, *etc.* Variability in percentage callus initiation was observed among genotypes of barley. The regeneration potentiality of different genotypes was hardly influenced by phytohormones, particularly during the initial stages of culture. But it retained very less potentiality for regeneration after a period of long sub-culturing when it was not supplemented with a particular combination of hormones to trigger the inbuilt potentiality of a particular genotype to regenerate. The morphogenetic ability still present after long subculturing was however sufficient enough for purposes like regeneration after *in vitro* selection (Chawla and Wenzel 1987a, 1987c).

Enzyme pattern of an organism changes during development and differentiation. Changes of isozyme patterns in samples of a particular organ or tissue during development is seen by the appearance and disappearance of individual isozymes. Such changes in isozymes suggest that genes involved in the synthesis of these enzymes are differentially activated in development. These can be used as markers for identification of cultivars (Moore and Collins 1983). In maize, changes in esterase and peroxidase isozymes have been associated with specific tissue (Scandalios 1969, Prasanna *et al.* 1983). Identification and characterisation of cultivars by isozymes of esterase, acid phosphatase and perioxidase have been reported in barley (Bassiri 1976) and in wheat (Bergmann and Mann 1973). With the development of shoot(s)/root(s) new isozymes appeared for all the three enzymes. Isozyme profile of young and old calluses showed the same pattern for peroxidase and acid phosphatase, but for esterase in old calli, the two isozymes of low intensity were absent. It can be inferred that when morphogenesis was induced in the undifferentiated tissue, it did not result in the induction of characteristic and conspicuous isozymes. It indicates that for

shoot and/or root development these isozymes are involved in differentiated tissue rather than in the induction of morphogenesis.

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