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THE EFFECT OF L-PROLINE ON SOMATIC EMBRYOGENESIS IN LONG-TERM CALLUS CULTURE OF *HORDEUM VULGARE**

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Long-term callus cultures of barley (*Hordeum vulgare* L.) with decreased embryogenic potential were used in testing the influence of L-proline on somatic embryogenesis. The addition of up to 10 mM L-proline to the induction medium increased both the frequency of embryoid formation and the number of subsequently regenerated plantlets. Most of the plantlets (82%), however, were albinos. Some callus lines retained embryoid and plantlet formation capacity after being in culture for more than 21 months.

Introduction

The establishment of long-term, high-regenerable cell lines in which plants regenerate through somatic embryogenesis seems to be a prerequisite for the use of tissue culture in cereal improvement. However, it was shown that the embryogenic potential declined rapidly during the first several subcultures. Only a few reports proved that embryogenesis in cereal cell culture could last about a year or more (Lu et al. 1981, Heyser et al. 1983, 1985).

The present paper describes the improvement of embryogenic potential in up to 21-month-old barley callus by using L-proline.

Material and Methods

Cell lines of two-row winter cultivars of *Hordeum vulgare* L. (Alkar and Slavonac), which had been established as described before (Rengel and Jelaska 1986), were used in present experiments.

* This paper is dedicated to Prof. Z. Devidé on the occasion of his 65th birthday.

Basal medium composed of macro- and microelements according to Murashige and Skoog (1962) supplemented with (mg l^{-1}): m-inositol 500, thiamine-HCl 5, pyridoxine-HCl 5, nicotinic acid 5 (MS), solidified with agar (10 g l^{-1}) was used throughout. Different growth regulators as well as sucrose in various concentrations were used according to experimental objectives. The duration of subcultures was 6 weeks.

The cultures were incubated in a growth chamber at 26±1° C exposed to artificial light of fluorescent lamps (spectral range 400—700 nm, 17 W m^{-2} and a light/dark cycle of 16/8 hours).

Results

Barley callus cultures were grown on an agar solidified MS medium supplemented with 4.5% sucrose, 7.8 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and 2.2 μM BA (6-benzyladenine) for eight subcultures (fourteen months of cultivation). Only calli from the eighth subculture that still generated embryoids, albeit with low frequency, were tested further.

Callus tissues of two cultivars (twelve lines each) were equally divided and randomly transferred to the same induction medium supplemented with 0, 2, 10 or 30 mM L-proline. Only NE (non-embryogenic*) parts of calli (deprived of any distinguishable embryoids) were used. Those calli were friable, composed of yellow somewhat translucent, and hard pieces. The influence of L-proline on the maintenance of embryoid formation capacity was tested during five subsequent subcultures. The addition of up to 10 mM L-proline to the induction medium both stimulated embryoid formation from NE callus tissue in higher percentage and retained the embryogenic potential longer in comparison with no L-proline supplied (Fig. 1; results on the cultivar Alkar were found to be similar to the results presented on the cultivar Slavonac).

The embryoids which emerged differred in appearance from those which were induced in previous subcultures (cf. Rengel and Jelaska 1986). No white or opaque parts of E (embryogenic*) callus could be observed. Distinct coleoptile and visible translucent scutellum were used as criteria for distinguishing the embryoids (Fig. 2 A). Embryoids were always inserted into the mass of either other embryoids or NE callus, and coleorrhiza was not observed.

Developed embryoids were transferred to the regeneration medium (MS + 2% sucrose + 1% agar + 3 μM TIBA /2, 3, 5-triiodobenzoic acid/). Some of the embryoids grew into green, while the majority of them developed into albino shoots and plantlets (Figs. 1 and 2 B-C). The ratio albino/green shoots and plantlets increased during subculturing (61, 86 and 100% in the tenth, eleventh and twelfth subculture, respectively). Albino shoots could not grow to maturity and their root system was either poorly developed or entirely absent. In order to overcome these problems other induction and regeneration media were tested in the thirteen and fourteen subcultures. Raising the BA concentration to 8.8 μM in the induction medium (25% of cultures produced embryoids) or sucrose to 12% (17% of cultures were embryogenic) did not cause any

* The terms »non-embryogenic« and »embryogenic« are used according to Nabors et al. (1983).

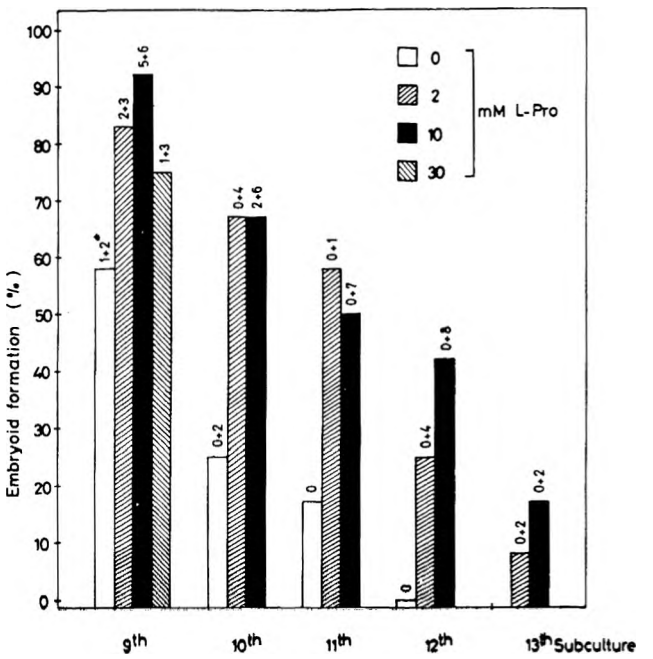

Fig. 1. Influence of L-proline on embryoid formation (% of calli with embryogenesis) in NE callus tissue and plantlet regeneration in subsequent subculture.

Induction medium: MS+4.5% sucrose+1% agar+7.8 μM 2,4-D+2.2 μM BA.

Regeneration medium: MS+2% sucrose+1% agar+3 μM TIBA.
Cultivar: Slavonac.

Sample size: 12 vials per treatment.

* No. of green+No. of albino plantlets

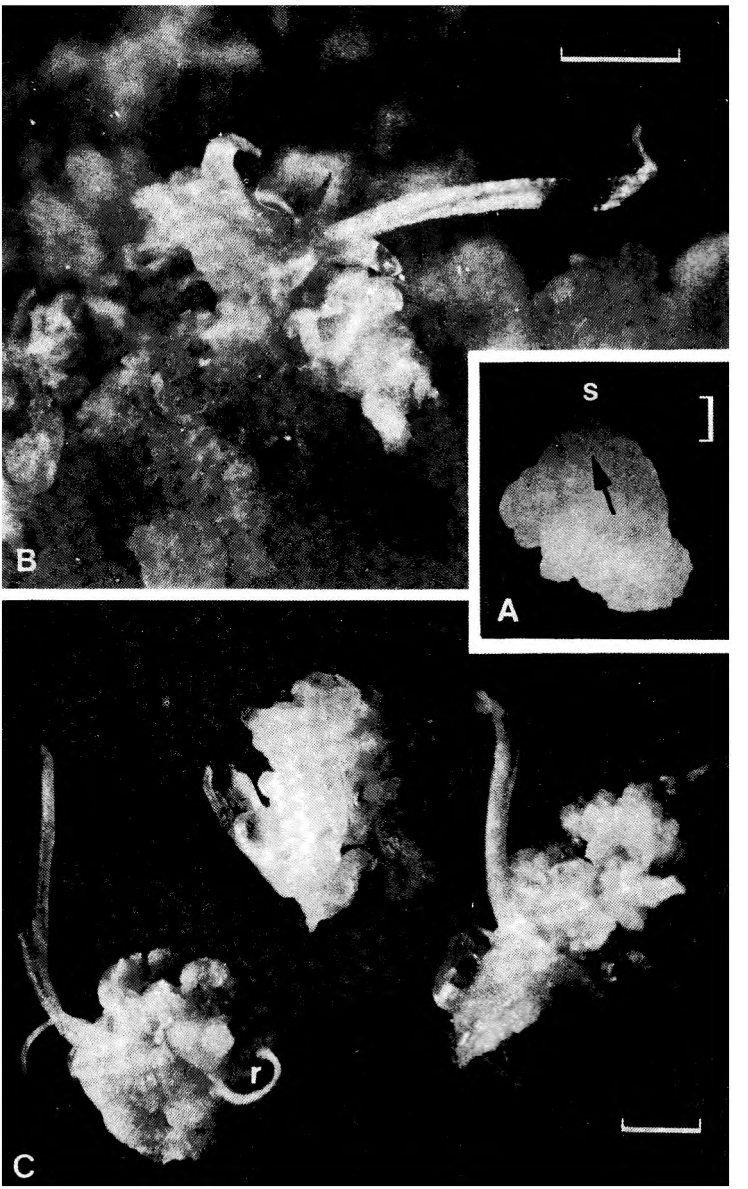

Fig. 2. Barley embryoid and plantlet formation in long-term callus culture. Cultivar: Alkar.
A. Embryoid with distinct coleoptile (arrow) and scutellum (s) developed on induction medium supplemented with 10 mM L-proline.
Bar = 0.1 cm.
B-C. Barley plantlets developed from embryoids on regeneration medium supplemented with 3 μM TIBA (r = root).
Bar = 1 cm.

significant improvement of embryoid formation in comparison with the medium supplemented with 2.2 μM BA, 4.5% sucrose and 10 mM L-proline (17% of cultures produced embryoids).

Several regeneration media were also tested. Plants did not regenerate when the basal medium (MS + 2% sucrose + 1% agar) was supplemented either with 0.4 μM abscisic acid or 4.7 μM kinetin. In the cases of either 5 μM gibberellic acid (GA_3) or no growth regulators used, development of only albino plants was achieved.

Discussion

Somatic embryogenesis in the cell culture of cereals seems to be an alternative to the sporadic plant regeneration by organogenesis. However, the reduction of embryogenic potential during subculturing presents the largest obstacle. Embryoid formation from long-term cultures of the family *Poaceae* was reported by Lu et al. (1981) and Gamborg et al. (1970) (18 and 24-month-old cultures, respectively).

We obtained somatic embryogenesis in barley cultures up to 21-month-old and some of them retained their embryogenic capacity even later. It is important to stress that all along the culturing only NE calli were subcultured on induction media and E callus and embryoids permanently arose from them. The interconversion of NE to E cells could be proposed. It is consistent with earlier observations (Nabors et al. 1983, Heyser et al. 1983, 1985, Rengel and Jelaska 1986). However, the existence of E cells (as »pockets«) in the mass of NE callus during successive passages could also be taken into account (as stated by Botti and Vasil 1983; but for a small number of passages). On the contrary, Street (1979) proposed, with respect to dicots, that E and NE cells formed two non-interconvertible populations established during culture initiation. Extensive cytological work is needed to clarify this doubt.

It appears from our results that the addition of L-proline to the induction medium could improve somatic embryogenesis in long-term barley callus. The stimulative effect of L-proline on embryoid formation from cultured anthers of triticale (Sozinov et al. 1981) as well as on somatic embryogenesis in callus cultures of *Medicago sitiva* (Stuart and Strickland 1984) and *Zea mays* (Amstrong and Green 1982, 1985) has been reported. However, Amstrong and Green (1985) stated that the stimulative effect of L-proline depended upon the inorganic nitrogen components of the medium. From their results it appears that the high inorganic nitrogen content in the medium (like in MS, Murashige and Skoog 1962) diminishes the positive effect of L-proline on somatic embryogenesis in maize. On the contrary, we obtained the stimulative effect of L-proline by using MS medium.

In the course of the experiments described here 19 green and 86 albino shoots and plantlets were regenerated. The very high percentage of albino shoots and plantlets (82%) seems characteristic of older cultures and was not observed in the same cultures of barley during the first several subcultures (4%; Rengel and Jelaska 1986) and from the fifth to eighth subcultures (10%; Rengel 1986). Gamborg et al. (1970) regenerated only albino plants from 24-month-old cultures of *Bromus inermis*. Kott and Kasha (1984) found that the majority of plants regenerated from cultures of haploid barley, ranging in age

from six weeks to thirty months, were albinos. On the contrary, no regenerated albino plants were obtained in up to 14-month-old callus cultures originating from barley mesocotyl (J e l a s k a et al. 1984).

Although we used different regeneration media in the experiments presented here, after a certain age of cultures, green plants were not produced. So, it is possible that long cultivation (with 2,4-D included in the medium) caused accumulation of genetic changes in cultured cells and affected both the viability of embryoids and the differentiation of plastids in plantlets regenerated. Moreover, long cultivation may cause adaptation to the heterotrophic nutrition to such an extent that it can interfere with both the normal plastid development and the crossing to the autotrophic way of living.

Further improvements in culturing conditions are needed to retain not only the capacity for embryogenesis but also the ability to regenerate normal green plants from long-term barley cultures.

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SAŽETAK

UTJECAJ L-PROLINA NA SOMATSKU EMBRIOGENEZU U DUGOTRAJNIM KALUSNIM KULTURAMA JEČMA (*HORDEUM VULGARE* L.)

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Dugotrajne kalusne kulture ječma (*Hordeum vulgare* L.) sa smanjenim embriogenim potencijalom korištene su za ispitivanje utjecaja L-prolina na somatsku embriogenezu. Dodatak do 10 mM L-prolina u indukcijsku podlogu povećao je ne samo učestalost nastanjanja embrioida nego i broj biljčica koje su naknadno regenerirane. Ipak, većina biljčica (82%) bila je albino. Neke kalusne linije zadržale su sposobnost stvaranja embrioida i biljčica i nakon što su kultivirane više od 21 mjesec.

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