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The effect of polyamines on the development of sugar beet protoplasts

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Abstract

The influence of the exogenous polyamines: putrescine, spermidine and spermine, on the frequency of protoplast divisions for 2 genotypes of sugar beet (*Beta vulgaris* L.) was analyzed. Protoplasts were cultured by the agarose disk method on Saunders and Doley medium supplemented with either hormones or polyamines, or hormones combined with polyamines. The latter supplement led to a statistically significant increase in plating efficiency. The improvement in division index was caused mainly by spermine.

Key words: Beta vulgaris, plating efficiency, protoplasts, putrescine, spermidine, spermine.

Introduction

Sugar beet (*Beta vulgaris* L.) protoplasts are considered a recalcitrant material with regard to their behaviour under *in vitro* conditions, and particularly their suitability for genetic manipulation within this species (Hall *et al.* 1993). The main difficulties in introducing protoplast-based modifications in practical breeding are related to problems in obtaining highly efficient division of protoplasts, as well as to the very limited capacity to regenerate plants in variable experimental systems (Pedersen *et al.* 1993, Hall *et al.* 1995).

In the last decade many efforts have been made to increase plating efficiency (PE) of sugar beet protoplasts obtained from different plant tissues, especially suspension

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Abbreviations: BAP - 6-benzylaminopurine, NAA - naphthaleneacetic acid, PAs- polyamines, PE - plating efficiency, Put - putrescine, SD - Saunders and Doley medium, Spd - spermidine, Spm - spermine.

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cultures and leaves (Szabados and Gaggero 1985, Bhat *et al.* 1985, Schlangstedt *et al.* 1992, 1994). The composition of the medium may influence PE to some extent. The kind and concentration of exogenous hormones are factors related to significant changes in the rate of division of both suspension-derived and mesophyll protoplasts of several sugar beet genotypes (Szabados and Gaggero 1985, Bhat *et al.* 1985, 1986). Very complex media such as KM8p (Bhat *et al.* 1985), as well as conditioned media (Szabados and Gaggero 1985), also had a stimulatory effect on protoplasts division. In some cases the application of antioxidants, such as *n*-propyl gallate, which increase the stability of protoplast membranes, seems to influence the number of viable protoplasts and indirectly the number of those capable of division (Jażdżewska *et al.* 1995). However, the most spectacular effect on sugar beet protoplast PE has been observed after their immobilization in agarose or alginate (Schlangstedt *et al.* 1992, Majewska-Sawka *et al.* 1994).

Among factors stimulating division of both cells and protoplasts of different plant species, polyamines are the most efficient (Pfosser *et al.* 1990, Tiburcio *et al.* 1993). The exact mechanisms of their action are only partially understood. Only few studies on protoplast-polyamine interactions deal with physiological changes during and immediately after isolation (Altman *et al.* 1977, Antognoni *et al.* 1995), and usually do not describe the long-term effect in protoplast cultures. Studies of the action of polyamine are further complicated by the fact that the effect of exogenous compounds differs depending on the genotype, the tissue and the content of endogenous polyamines (Evans and Malmberg 1989, Ye *et al.* 1994).

The identification of external factors which may improve protocols for sugar beet protoplast culture seems to be a crucial prerequisite for the successful application of protoplast-based techniques for the genetic manipulation of this species. We report the influence of exogenous, natural polyamines on division and the development of suspension-derived protoplasts from two sugar beet genotypes, used as our starting material for asymmetric somatic hybridization (Jażdżewska *et al.* 1995).

Materials and methods

Plants: Cytoplasmic male sterile diploid line 491D (Maribo Breeding Company, Denmark) and male fertile diploid pollinator JP3 (Plant Breeding and Acclimatization Institute, Poland) were studied. Callus cultures and callus-derived suspensions were initiated and maintained as described previously (Jażdżewska *et al.* 1995).

Isolation and culture of protoplasts: Protoplasts were isolated 2 or 3 d after suspension subculture. They were purified by filtration of a protoplast-enzyme mixture through 60 and 40 μm nylon meshes, washed twice in salt solution (Frearson *et al.* 1973) with 0.6 M mannitol, and centrifuged at 75 *g* for 5 - 10 min. Protoplasts were then plated at a density of 1.0 - 1.5 × 10^5 cm^{-3} and cultured in modified SD medium (Saunders and Doley 1986) solidified with 1.25 % agarose (*FMC Bioproducts*, Rockland, USA) at 25 °C in the dark. The influence of exogenous

hormones and polyamines was determined by comparing plating efficiencies of protoplasts cultured in SD medium supplemented with one of the following:

A - 5 μM naphthaleneacetic acid (NAA) and 2 μM 6-benzylaminopurine (BAP) (control),
B - 44 μM each of polyamine: spermidine (Spd), spermine (Spm) and putrescine (Put) (*Sigma Chemical Co.*, St. Louis, USA)
C - 5 μM NAA, 2 μM BAP and 44 μM Put, Spd and Spm,
D - 5 μM NAA, 2 μM BAP and 44 μM Put,
E - 5 μM NAA, 2 μM BAP and 44 μM Spd,
F - 5 μM NAA, 2 μM BAP and 44 μM Spm.

PE was expressed as the percentage of protoplasts which gave rise to callus microcolonies after 2 weeks of culture.

Statistical analysis: Each experiment was replicated 3 - 7 times. The numbers of initially plated protoplasts and those which developed into calli were counted in 20 square areas of 0.58 mm^2 chosen at random. Subsequently, the percentage indexes of protoplast-derived calli were determined based on all individual data. These values were trigonometrically transformed by arc $\sin\sqrt{x}$ and compared by Tuckey test. Least significant differences were estimated for $P = 0.001$ or 0.05.

Results and discussion

The simultaneous addition of 44 μM Put, Spd and Spm to SD medium containing 5 μM NAA and 2 μM BAP resulted in a conspicuous stimulation of the rate of protoplast division and number of microcolonies formed in comparison with control cultures (Figs. 1, 2; Table 1). The positive influence of PAs combined with hormones was observed in both genotypes tested, and the differences between variants A and C were statistically significant. The polyamines alone, when not combined with hormones, had a detrimental effect on JP3 protoplast development compared to control cultures (Table 1).

Table 1. The effect of exogenous hormones and polyamines on plating efficiencies [%] of sugar beet protoplasts. Significant differences were found between means with different letters (for transformed data $y = \text{arc sin}\sqrt{x}$), $P = 0.001$

Medium*	Plating efficiency 491D	JP3
A - hormones	30.0 b	18.3 b
B - polyamines	25.4 b	7.7 c
C - hormones combined with polyamines	42.6 a	30.5 a

(* see Materials and Methods)

The increase in PE after polyamine application probably results from increases in both DNA synthesis and mitotic index; this action of PAs is widely known in plant cells (Kaur-Sawhney *et al.* 1980, Minocha *et al.* 1991). Few reports are available

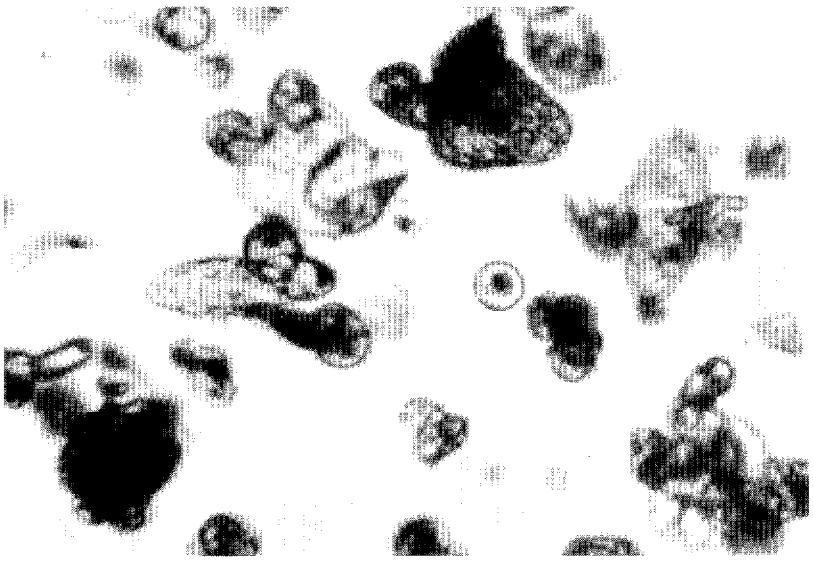

Fig. 1. Callus microcolonies after 2 weeks of culture in medium with hormones (medium A).

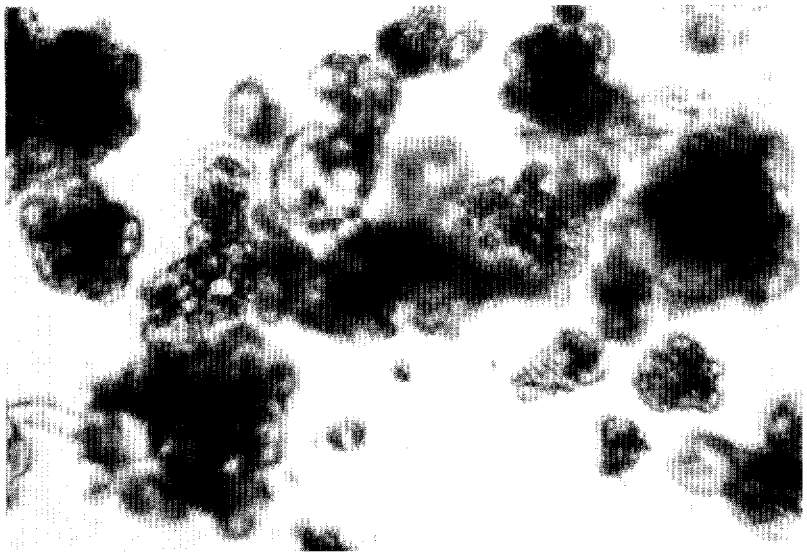

Fig. 2. Callus microcolonies after 2 weeks of culture in medium with hormones and three polyamines (medium C).

which analyze the influence of exogenous polyamines on protoplast isolation and development. Kaur-Sawhney *et al.* (1980) stated that treatments with 1 mM Spd or Spm significantly increased nuclear divisions in oat leaf protoplasts. Similarly, sweet potato petiole protoplasts divided most efficiently in the presence of polyamines (Eilers *et al.* 1988). Tiburcio *et al.* (1986) improved the viability of oat mesophyll protoplasts by a two-fold increase in Spm content. Altman *et al.* (1977) found that exogenous polyamines stabilize protoplasts against lysis and increase rates of protein and RNA synthesis.

The involvement of polyamines in stabilization of the protoplast membrane is now well established (Altman *et al.* 1977, Galston 1983). Two possible mechanisms have been proposed. The first involves the PAs-dependent decrease in RNase and protease activities, and the second the decrease in ethylene production due to inhibition of ACC synthase (Tiburcio *et al.* 1993, Hagège *et al.* 1994). In control cultures, *i.e.* in the absence of exogenous polyamines, sugar beet protoplasts show very high viability even several days after isolation (Jazdzewska *et al.* 1995). Membrane stabilization therefore seems not to be the main factor involved in increase in PE. Polyamine-related inhibition of ethylene synthesis is therefore the more likely explanation.

Table 2. The effect of individual polyamines on plating efficiencies [%] of sugar beet protoplasts. Significant differences were found between means with different letters (for transformed data $y = \text{arc sin } \sqrt{x}$), $P = 0.05$ for 491D and 0.001 for JP3.

Medium	Plating efficiency	
	491D	JP3
A - hormones	25.5 b	20.5 b
D - hormones + Put	33.9 ab	27.6 b
E - hormones + Spd	41.9 a	24.7 b
F - hormones + Spm	42.6 a	51.1 a

To identify which of the three polyamines plays the most important role in increasing DNA synthesis and stimulating mitotic divisions in sugar beet protoplasts, cultures with each individual PAs were carried out. The response of both genotypes was similar when Spm was added into the medium. This polyamine caused a significant increase in division frequency (Table 2). When Put or Spd was added, the 491D was highly responsive genotype, showing the increase in PE (Table 2). Genotype JP3 was less sensitive to exogenous Put or Spd in the concentrations used: PE in control medium and in media containing Put or Spd remained at similar level (Table 2). Our results thus show that Spm is the compound that most actively increased the index of mitotic division in sugar beet protoplasts. This is in agreement with Galston's theory, according to which different PAs are related to different processes, and cell divisions are correlated with Spm and Spd level (Galston 1983). Spermine has also been shown to stabilize oat protoplasts much better than Spd and Put, and to decrease senescence in oat leaves (Galston 1983). Both observations may be related to the fact that Spm has a stronger inhibitory effect on both ACC synthase

activity and ethylene production than other polyamines (Tiburcio *et al.* 1993, Hagège *et al.* 1994).

In addition, our results show that polyamines alone, when not combined with hormones, do not induce mitotic divisions to the same extent as hormones do. It is therefore reasonable to assume that PAs interact with either auxin or cytokinin, or both, and strongly stimulate the cells to proliferate. The exact molecular mechanism of hormone-polyamine interaction is not known, but some possibilities have been put forward during the last few years. Havelange *et al.* (1996) emphasized that PAs do not act alone in the regulation of cell proliferation, and noted that cytokinins also contribute to this process. The export of Put from mature leaves of *Sinapis alba* is correlated with the simultaneous transport of this hormone (Havelange *et al.* 1996). Moreover, the production of PAs is activated by cytokinins in some plant systems. It has been proposed that both hormones and PAs play a role in signal transduction pathways (Ye *et al.* 1994) and influence the metabolism of phosphatidylinositols (Tiburcio *et al.* 1993).

The mechanisms discussed above for the increase in mitotic divisions after polyamines treatment should be verified by experiments aimed at determining differences in the activity of enzymes related to both polyamines and ethylene production in control and induced protoplasts.

References

Altman, A., Kaur-Sawhney, R., Galston, A.W.: Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. - Plant Physiol. **60**: 570-574, 1977.

Antognoni, F., Pistocchi, R., Casali, P., Bagni, N.: Does calcium regulate polyamine uptake in carrot protoplasts? - Plant Physiol. Biochem. **33**: 701-708, 1995.

Bhat, S.R., Ford-Lloyd, B.V., Callow, J.A.: Isolation of protoplasts and regeneration of callus from suspension cultures of cultivated beets. - Plant Cell Rep. **4**: 348-350, 1985.

Bhat, S.R., Ford-Lloyd, B.V., Callow, J.A.: Isolation and culture of mesophyll protoplasts of garden, fodder and sugar beets using a nourse culture system: callus formation and organogenesis. - J. Plant Physiol. **124**: 419-423, 1986.

Eilers, R.J., Sullivan, J.G., Skirvin, R.M.: Analyzing the effects of exogenous polyamines and growth regulators on plating efficiency of sweet potato protoplasts using a central composite test design. - Plant Cell Rep. **7**: 216-219, 1988.

Evans, P.T., Malmberg, R.L.: Do polyamines have roles in plant development. - Annu. Rev. Plant Physiol. Plant mol. Biol. **40**: 235-269, 1989.

Frearson, E.M., Power J.B., Cocking E.C.: The isolation, culture and regeneration of *Petunia* leaf protoplasts. - Dev. Biol. **33**: 130-137, 1973.

Galston, A.W.: Polyamines as modulators of plant development. - BioScience **33**: 382-388, 1983.

Hall, R.D., Pedersen, C., Krens, F.A.: Progress towards the development of a general somatic hybridization protocol for *Beta*. - J. Sugar Beet Res. **30**: 275-289, 1993.

Hall, R.D., Verhoeven, H.A., Krens, F.A.: Computer assisted identification of protoplasts responsible for rare division events reveals guard-cell totipotency. - Plant Physiol. **107**: 1379-1386, 1995.

Hagège, D., Kevers, C., Geuns, J., Gaspar, T.: Ethylene production and polyamine content of fully habituated sugar beet calli. - J. Plant Physiol. **143**: 722-725, 1994.

Havelange, A., Lejeune, P., Bernier, G., Kaur-Sawhney, R., Galston, A.W.: Putrescine export from leaves in relation to floral transition in *Sinapsis alba*. - Physiol. Plant. **96**: 59-65, 1996.

Jaździewska, E., Niklas, A., Majewska-Sawka, A.: Progress towards sugar beet improvement through somatic hybridization. 1. Inactivation of nuclei and cytoplasm in donor and recipient protoplasts. - Acta Soc. Bot. Pol. **64**: 341-347, 1995.

Kaur-Sawhney, R., Flores, H.E., Galston, A.W.: Polyamine-induced DNA synthesis and mitosis in oat leaf protoplasts. - Plant Physiol. **65**: 368-371, 1980.

Minocha, R., Minocha, S.C., Komamine, A., Shortle, W.C.: Regulation of DNA synthesis and cell division by polyamines in *Catharanthus roseus* suspension cultures. - Plant Cell Rep. **10:** 126-130, 1991.

Majewska-Sawka, A., Nakashima, H., Mori, K.: Isolation and culture of suspension-derived protoplasts of *Beta vulgaris* L. - Biol. Plant. **36**: 9-13, 1994.

Pedersen, Ch., Hall, R.D., Krens, F.A.: Petioles as the tissue source for isolation and culture of *Beta vulgaris* and *B. maritima* protoplasts. - Plant Sci. **95:** 89-97, 1993.

Pfosser, M., Königshofer, H., Kandeler, R.: Free, conjugated, and bound polyamines during the cell cycle of synchronized cell suspension cultures of *Nicotiana tabacum*. - J. Plant Physiol. **136**: 574-579, 1990.

Saunders, J.W., Doley W.P.: One step shoot regeneration from callus of whole plant leaf explants of sugar beet lines and a somaclonal variant for *in vitro* behaviour. - J. Plant Physiol. **124**: 473-479, 1986.

Schlangstedt, M., Hermans, B., Zoglauer, K., Schieder, O.: Culture of sugar beet (*Beta vulgaris* L.) protoplasts in alginate - callus formation and root organogenesis. - J. Plant Physiol. **140**: 339-344, 1992.

Schlangstedt, M., Zoglauer, K., Lenzner, S., Hermans, B., Jacobs, M.: Improvement of sugar beet (*Beta vulgaris* L.) protoplast culture: leaf petioles as a protoplast source. - J. Plant Physiol. **143**: 227-133, 1994.

Szabados, L., Gaggero, C.: Callus formation from protoplasts of a sugarbeet cell suspension culture. Plant Cell Rep. **4**: 195-198, 1985.

Tiburcio, A.F., Kaur-Sawhney, R., Galston, A.W.: Polyamine metabolism and osmotic stress. II. Improvement of oat protoplasts by an inhibitor of arginine decarboxylase. - Plant Physiol. **82**: 375-378, 1986.

Tiburcio, A.F., Campos, J.L., Figueras, X., Besford, R.T.: Recent advances in the understanding of polyamine functions during plant development. - Plant Growth Regul. **12**: 331-340, 1993.

Ye, X.S., Avdiushko, S.A., Kuc, J.: Effects of polyamines on *in vitro* phosphorylation of soluble and plasma membrane by proteins in tobacco, cucumber and *Arabidopsis thaliana*. - Plant Sci. **97**: 109-118, 1994.