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Paclobutrazol stimulates bud regeneration in *Solanum tuberosum* L. primary explant cultures

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Abstract

The growth retardant paclobutrazol (PBZ) inhibited stem internode growth of *in vitro* cloned potato plants. The extent of growth inhibition caused by 10^{-9} M PBZ in Murashige and Skoog medium was genotype-specific, varying between 10 - 60 % of the stem growth of untreated controls in ten cultivars examined. An increase in percentage of *de novo* bud regenerating stem internode segments (SIS) as well as in the total number of buds per explant was observed in SIS taken from PBZ pretreated plants. PBZ applied directly into the regenerative media had no stimulatory effect on the regeneration process. We assume that the enhancing effect of PBZ on regeneration may be attributed to its interaction with cytokinin metabolism.

Additional key words: growth regulators, paclobutrazol, potato, regeneration ability.

Introduction

The low regenerative capacity of plant explants represents one of the main limits on the practical use of molecular and cell techniques for construction of new genotypes of some crops, including potato. Within the set of more than 30 potato cultivars tested in our laboratory, great differences in regeneration ability, *i.e.* in *de novo* regeneration of buds in potato primary explant cultures, have been observed (Opatrná *et al.* 1990).

To overcome regeneration blocks in particular potato genotypes, various approaches have been compared, focused mainly on effects upon natural hormonal balance in the explant tissues. Among other techniques, these have included modifications of the hormonal composition of culture media and insertion of bacterial oncogenes (Webb *et al.* 1983, Wheeler *et al* 1985, Coleman *et al.* 1990,

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Abbreviations: BAP - 6-benzylaminopurine, 2,4-D - 2,4-dichlorophenoxyacetic acid; 2ip - isopentenyladenine; IAA - indoleacetic acid; NAA - naphthaleneacetic acid.

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Ovesná *et al.* 1993). Various auxins (IAA, NAA, 2,4-D) and cytokinins (BAP, kinetin, zeatin, 2iP) have frequently been used to stimulate bud initiation and/or subsequent development.

More recently, the regulatory effects of some growth retardants on growth and development of *in vitro* propagated plants were reported (Ziv *et al.* 1986, Ziv 1989, Ziv and Ariel 1991, Novák 1994, Werbrouk and Debergh 1995).

The growth retardant paclobutrazol [P333; 1-(4-chlorophenyl)-4,4-dimethyl-2-(1-H-1,2,4-triazol-1-yl)-pentan-3-ol] is one of a group of triazole fungicides in which pronounced growth-retarding effect have been found (Jung *et al.* 1987, Rademacher 1991). They inhibit stem growth due to a reduction of gibberellic acid biosynthesis (Dalziel and Laurence 1984, Hedden and Graebe 1985, Grossmann 1990, Rademacher 1991). Later a more pleiotropic effect of paclobutrazol (PBZ) on plant metabolism has been postulated, including the biosynthesis of sterols, abscisic acid and cytokinins (Rademacher 1991, Buta and Spaulding 1991, Pinhero and Fletcher 1994).

The aims of the present work were to examine both the effect of PBZ on growth of stock plants and on the *de novo* bud regeneration of explants derived from PBZ treated plants. Simultaneously the morphoregulatory effect of PBZ applied directly into regenerative medium was investigated. The genotype specific regenerative responses to PBZ were characterized.

Materials and methods

The experimental system of *in vitro* cultured stem internode sections (SIS) excised from stock plants of various *in vitro* cloned potato cultivars was used as described by Ovesná *et al* 1993. A two-step regeneration method was applied (Opatrný and Müllerová 1986) in which SIS excised from donor plants were cultured at first on an inductive MS medium containing BAP (2.1 mg dm^{-3}), IAA (0.1 mg dm^{-3}) and adenine (40 mg dm^{-3}) for three weeks. After this they were transferred on to MS medium with IAA (0.1 mg dm^{-3}) only. On this medium the already initiated meristematic structures developed within 2 - 6 weeks into buds and shoots. PBZ dissolved in water and filter sterilized was applied at various concentrations either to culture media for stock plants (MS without growth regulators) or directly into the regenerative medium.

To characterize growth and regenerative response of the material, various parameters were followed, in particular stem height and leaf number of stock plants which developed from nodal segments containing axillary buds. The number of buds/shoots per explant and the percentage of regenerating explants served as criteria of SIS regeneration. The dynamics of bud formation was estimated. The response of 50 explants per treatment was evaluated in individual experiments. Four independent experiments were carried out with each set.

Results

PBZ-induced growth retardation of the donor plants: The possibility of improvement in regeneration ability of stem explants due to PBZ treatment was tested in ten cultivars, possessing different degrees of natural regeneration responsiveness under standard conditions: cvs. Karin, Šárka, Xenie and Ostara exhibited a low regeneration capacity in contrast to the cvs. Desirée, Kamýk and Lada, which were highly responsible (Fig. 2*A,B*, control variants).

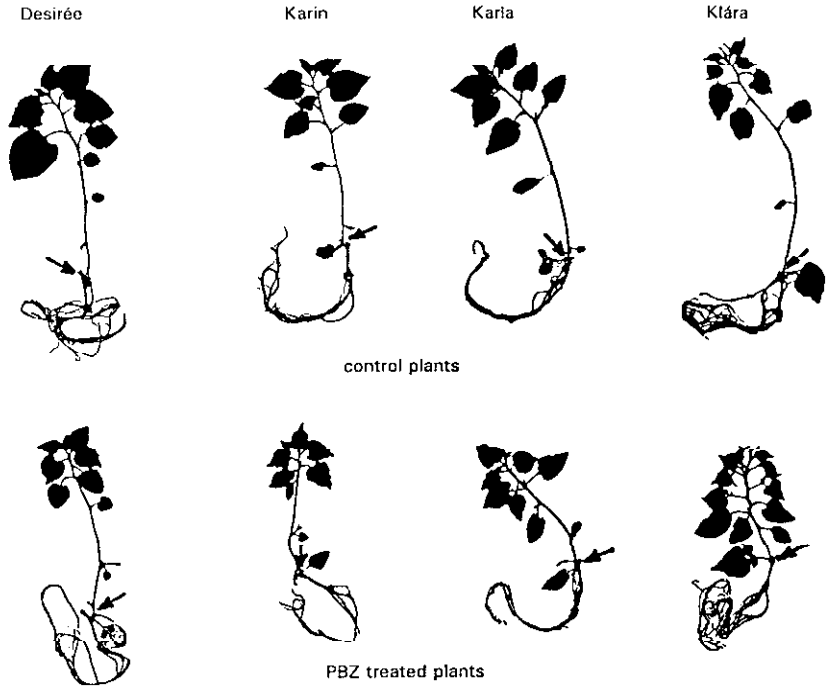

Fig.1. Paclobutrazol-induced changes in stem growth and morphology in *in vitro* cloned plants (5-weeks old) of potato cultivars Desirée, Karin, Karla and Klára. *Arrows* indicate the positions of original axillary buds.

Table 1. Stem height of PBZ treated plants as a percentage of untreated controls (= 100 %). No - cultivar number used in following figures.

No.	Cultivar	Stem height [%]	No.	Cultivar	Stem height [%]
1	Šárka	57.3	6	Karla	62.4
2	Klára	45.6	7	Kamýk	81.9
3	Lada	66.5	8	Zlata	98.7
4	Desirée	88.6	9	Xenie	71.4
5	Karin	55.6	10	Ostara	60.6

As the next step, the growth sensitivity of *in vitro* cloned donor plants to PBZ was evaluated. The concentration of PBZ was chosen in such a way as to inhibit only partly the stem growth of stock plants. Between 40 - 70 % retardation as compared with untreated controls was induced by 10^{-9} M PBZ. The response was highly genotype specific (Fig.1, Table 1): the cvs. Klára, Karin, and Šárka exhibited much higher sensitivity than cvs. Zlata, Kamýk and Desirée, the remaining four cvs. showed average levels of retardation. The extreme differences between cv. Zlata and cv. Klára reached up to one order of concentration - 40 % inhibition was achieved by 10^{-9} M PBZ in cv. Klára, but 10^{-8} M in cv. Zlata. In contrast with stem growth inhibition, the number of leaves was not influenced by these concentrations of PBZ in any genotype.

The effect of PBZ on *de novo* bud regeneration in SIS explants: For the *de novo* shoot bud regeneration from SIS taken from retarded and/or control plants, a standard two step regeneration system was used. Both increase in the percentage of regenerating explants (*Fig. 2A*) and in the number of buds and shoots per explant (*Fig. 2B*) were

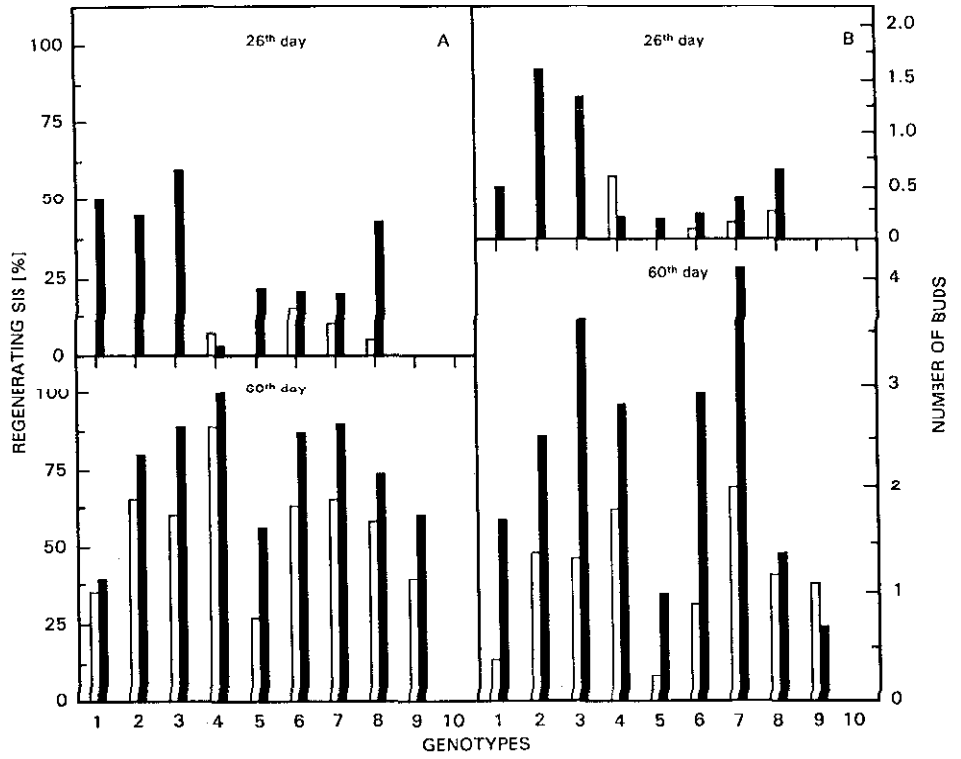

Fig. 2. Paclobutrazol-stimulated *de novo* bud regeneration in SIS of different potato cultivars (Nos. 1 - 10, see Table 1), expressed either as total number of regenerated buds per SIS (*A*) or as the percentage of regenerated SIS (*B*). SIS of control plants - *white columns*, SIS of PBZ-treated plants - *black columns*.

registered in SIS taken from PBZ treated plants of almost all genotypes, with the only exception of cv. Ostara, an extreme non-regenerating material. Marked acceleration of regeneration process was observed in most genotypes. The first buds already differentiated in the course of two weeks cultivation.

SIS taken from PBZ retarded plants exhibited generally a higher viability, decreased incidence of necrosis or vitrification and they preserved a high chlorophyll content (data not given) during cultivation, even when this was prolonged to more than 12 weeks. The total number of regenerated shoots might be limited by correlative growth inhibition between earlier and later differentiated buds and shoots. These difficulties can be partly overcome either by continuous removal of regenerated shoots and/or by explant segmentation with subsequent segment subculturing on fresh media.

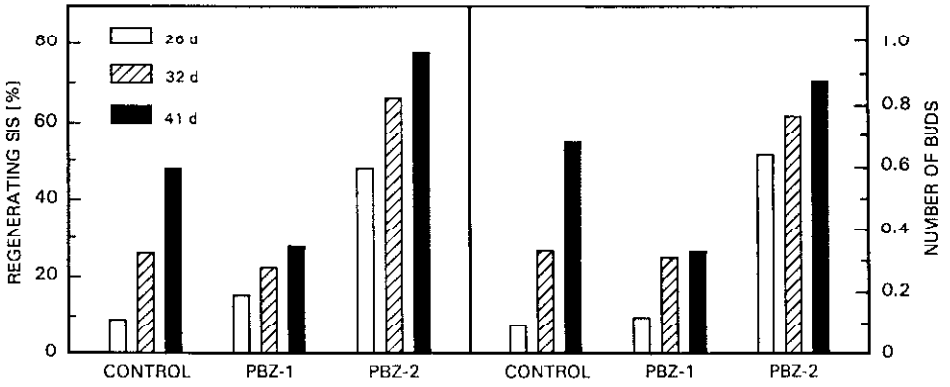

Fig. 3. The effect of PBZ on bud regeneration in dependence on the mode of its application. Control - SIS from non-treated plants of cv. Klára cultured on standard regenerative medium, PBZ-1 - SIS from non-treated plants cultured on PBZ (10^{-9} M) containing regenerative medium, PBZ-2 - SIS from PBZ (10^{-9} M) pretreated plants cultured on standard regenerative medium.

The application of this PBZ-pretreatment method to increase regeneration efficiency *in vitro* was, however, accompanied by one serious technical problem, *i.e.* by strong shortening of internodes. As mentioned above, the effect of PBZ treatment was highly dependent both on the concentration used and on genotype-specific sensitivity. A concentration of 10^{-9} M PBZ applied in culture medium for 3 - 4 weeks was the most convenient way to treat stock plants and to increase their SIS regeneration effectiveness. In the case of some cultivars it was not possible to excise more than two SIS per plant in comparison with five to six from control plants. We tested an alternative experimental strategy to overcome this obstacle, excising SIS from PBZ non-treated plants and applying PBZ directly into the regeneration medium. This treatment was compared both with the response of segments explanted from PBZ-retarded plants and with control segments explanted from untreated plants. All three types of explants regenerated on medium without retardant (Fig. 3). According to our observations PBZ applied into the regenerative medium did not significantly influence either the extent (number of buds, percentage of regenerating

explants) or the rate of bud primordia initiation in SIS. The presence of PBZ in regenerative media only inhibited the shoot growth of regenerated buds.

Discussion

Cultivar-specific sensitivity to PBZ: We found a wide range of cultivar responses to the effects of PBZ (growth inhibition of *in vitro* cloned plantlets and stimulation of bud regeneration from SIS explants). The observation of highly genotype specific growth-retarding effects of PBZ corresponds to our previous finding with other retardants: Retacel (CCC) and Alar (Svojšová 1991, Novák 1994). Of these PBZ exhibited the highest effect on stem growth at the lowest concentration applied (10^{-9} - 10^{-8} M), Alar showed a less inhibitory effect (10^{-4} to 5×10^{-4} M). Retacel inhibited stem growth only at a concentration of 10^{-3} M and higher. However, we did not find any common tendency in the sensitivity of 9 individually tested cultivars to all these three kinds of inhibitors of GA_3 synthesis. For instance, stock plants of cv. Klára and Lada, which both usually tend to extreme elongation of stem internodes under the conditions of test-tube culture, exhibited the minimal sensitivity to Retacel treatment. On the contrary cv. Lada was retarded by PBZ to a moderate extent and cv. Klára was the most sensitive to PBZ.

It is also difficult to trace more general correlations between the extent of the growth retarding effect of PBZ on the donor plants and on the corresponding regenerative response of the excised SIS. Four of ten cultivars tested - *i.e.* cvs. Xenie, Karla, Desirée, and in particular Zlata, exhibited the lowest growth retardation response (height of PBZ-treated plants being over 70 % of the controls). Of them cvs. Zlata and Xenie have shown the lowest regenerative response, but only in the value "bud number per SIS". Similary, the most stem growth sensitive cultivars, in particular cvs. Šárka and Karin, exhibited the highest PBZ-induced increase of SIS regeneration only in the value "bud number per SIS", the PBZ effect on the frequency of regenerating SIS was again comparable with the response of other cultivars.

Mode of PBZ action: Bud regeneration was stimulated only when PBZ was applied to donor plants. This indicates that either its sufficiently high endogenous level - reflecting its accumulation during donor plant cultivation - is necessary from the very beginning of the incubation of explants or that the metabolism of donor plants is affected by PBZ in the direction of increasing regenerative competency of SIS. We did not analyze the PBZ (PBZ-metabolites) content in plant tissue during our experiments, and found no relevant data in the literature.

Stem growth retardation of PBZ treated plants combined with normal leaf development resulted in four-fold higher starch accumulation (Grospietsch, unpublished results) in comparison with control plants. There is no doubt about the important role of both saccharide metabolism and transport (sink/source relationship) in potato morphogenesis both *in vivo* and *in vitro*. Detailed analysis of starch and endogenous saccharide content as well as their fluctuation during the regeneration process are currently under investigation.

As mentioned above, PBZ treated plants probably undergo changes in biosynthetic pathways of gibberellins, abscisic acid, cytokinins and sterols - which may result in better SIS regeneration.

Surprisingly, the direct PBZ application into the regeneration media had no positive effect on regeneration process of our potato SIS explants. This result contradicts to some extent the observations of Ziv (1989, 1990), Ziv *et al.* (1986) and Ziv and Ariel (1991). They reported the effect of different growth retardants - including the triazole compounds PBZ and ancymidol - on various ornamental plants propagated *in vitro* (ferns, philodendrons, gladioli) using liquid cultures in shakers and bioreactors. PBZ applied in the culture media positively affected proliferation and development of shoot clusters, inhibited leaf growth and decreased vitrification. It is difficult to conclude to what extent organ initiation itself or only further organ development were affected. The PBZ effect was assumed to be mostly mediated through elevated concentrations of cytokinins (Fletcher and Hofstra 1986, Grossman *et al.* 1987, Izumi *et al.* 1988). More recently Werbrouck and Debergh (1995) referred to strong synergistic interactions between some imidazole fungicides, structurally related to PBZ, and exogenously applied cytokinin (BAP). Bud induction by BAP was dramatically enhanced by imidazole in micropropagated *Spatiphyllum floribundum*. No pronounced changes in endogenous levels of cytokinins and metabolism of BAP were detected. Amongst alternative hypotheses the influence of these fungicides on cytokinin receptors was proposed.

The key role of cytokinins in regulation of regenerative responses of various potato cultivars have been repeatedly demonstrated in our previous studies (Ovesná *et al.* 1993). For this reason we can expect that PBZ-induced modification of their endogenous levels and ratios, either in a qualitative or quantitative manner, may explain the above-mentioned behaviour of SIS cultures. However, direct biochemical analysis is necessary to verify this hypothesis.

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