BULG. J. PLANT PHYSIOL., 1995, 21(1), 84–89

In vitro SCREENING FOR RESISTANCE TO PROLINE ANALOGUES IN ORCHARDGRASS *Dactylis glomerata* L.

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Received February 24, 1995

Summary. Plant regeneration capacity of orchardgrass leaf explants cultured *in vitro* in the presence of 0.01, 0.1, 1.0 and 10.0 mM hydroxyproline (Hyp) or azetidin-2-carboxylic acid (A2CA) was determined. Hyp inhibited to a lower extent than A2CA formation of somatic embryos, respectively plants. Hyp also induced relatively insignificant changes of free amino acid content in the leaves of the regenerants. Overproduction (3–10 times) of lysine, serine, arginine was detected in 3 plants obtained after treatment with 0.01 mM A2CA. The intracellular free proline content estimates suggest different mechanisms of resistance to proline analogues.

Key words*:* amino acids, *Dactylis glomerata* L., proline analogues, somatic embryogenesis

Abbreviations*:* A2CA — azetidin-2-carboxylic acid; Hyp — hydroxyproline

Introduction

In vitro screening and selection techniques have several potential advantages over whole plant screening in glasshouse or field situations (Duncan and Widholm, 1989; Dracup, 1991).

Resistance to inhibitory concentrations of amino acids or their analogues has been widely used to select variant cell lines and mutants. In cereal systems this approach has been followed to increase the synthesis of amino acids which are limiting in cereal grain (lysine, threonine, tryptophan) or involved in stress metabolism (proline). Furthermore, amino acid analogues can be used as tools for the study of amino acid metabolism (Bright, 1985).

The advantage–disadvantage balance of *in vitro* techniques in particular situations changes with the type of systems involved, especially when these methods are applied to recalcitrant species as cereals and grasses. In our experiments we used young leaf tissues from a highly embryogenic genotype of *Dactylis glomerata* L. (orchardgrass), kindly provided by Prof. B. V. Conger (University of Tennessee, USA). This genotype offers under definite culture conditions a reliable system for plant regeneration through direct (without callus phase) or indirect somatic embryogenesis.

The effect of two natural proline analogues – hydroxyproline (Hyp) and azetidin-2-carboxylic acid (A2CA) – on plant regeneration ability of orchardgrass leaf explants and free amino acid contents in regenerants was investigated.

Materials and Methods

Plant material. Leaf explants were chosen and cultured according to Conger et al. (1983). Briefly, segments (2–3 mm long) from the basal portions of the innermost two leaves were sterilized and plated on 0.8% agar SH medium (Schenk and Hildebrandt, 1972). The medium was supplemented with 6.6 $g.l^{-1}$ 3,6-dichloro-o-anisic acid (a synthetic auxin) for induction of callus and somatic embryo formation.

Evaluation of proline analogues effect. 0.01, 0.1, 1.0 and 10.0 mM filter sterilized Hyp and A2CA were added to the already autoclaved SH medium to test the influence of each proline analogue. After 4 weeks growth at 25 °C, in the dark the explants with formed callus and/or somatic embryos were transferred onto SH medium without auxin and analogues for plant regeneration. The culture conditions were 25 °C, 2000 lx light intensity and 16/8 h photoperiod. The average number of green, vigorous plantlets obtained from two independent experiments (in five replications each) was determined. After the plants had developed a root system they were transferred to soil in pots and maintained in a growth chamber.

Amino acid analysis. Extracts from the youngest leaf parts (capable for response under *in vitro* conditions) and from differentiated ones of 4-month-old plants were prepared according to Shiomi and Hori (1973). The intracellular pool of free amino acids was determined with an auto amino acid analyzer.

Protein assay. Soluble protein contents of the leaf parts, described above, were measured by Bradford's protein assay (1976).

Results and Discussion

Proline accumulating plant tissue culture cells have been isolated by their ability to grow in toxic concentrations of proline analogues (Lea and Forde, 1994). *In vitro* cultivation of orchardgrass leaf explants in the presence of Hyp or A2CA provides a

possibility for screening of mesophyll cells with naturally ocurring differences in sensitivity to growth inhibition produced by proline analogues. A part of these cells are competent for embryogenesis and they could produce somatic embryos, respectively plants, resistant to the applied agent probably due to some alterations of amino acid metabolism.

The inhibitory effect of proline analogues on plant regeneration capacity of explants is demonstrated in Fig.1. It is evident that Hyp influences formation of somatic embryos and their conversion to whole differentiated plants to a lower extent than the other analogue tested. The inhibition produced by 0.01 and 0.1 mM A2CA

Fig. 1. Inhibitory effect of Hyp and A2CA on plant regeneration ability of orchardgrass leaf explants

was similar, but a higher concentration blocked completely direct somatic embryogenesis. Apparently, there are no permeability barriers in the cells of orchardgrass leaf explants as their proliferation and growth were completely stopped by 10.0 mM Hyp or 1.0 mM A2CA. The effect of analogues on cell metabolism may be due to either proline synthesis inhibition through a “false end-product feed back” mechanism or functional impairment of proteins containing analogue residues in the place of proline (Mori et al., 1989).

At the concentrations applied Hyp induced relatively insignificant changes in the free amino acid content of orchardgrass leaves. An increased amino acid pool was detected in three of regenerants obtained from the explants cultured in the presence

Table 1. Effect of 0.01 mM A2CA on soluble protein and amino acid contents in plant leaves (per gram fresh weight)

Regenerant	Leaf parts	Soluble protein content (μg.g^{-1} FW)	Total amino acid content (nmol.g^{-1} FW)
Control	base	155.2	10424.8
	tip	250.5	16280.0
A-1	base	151.1	30856.0
	tip	230.8	21623.2
A-2	base	136.4	25893.0
	tip	236.3	16280.0
A-5	base	165.0	44455.7
	tip	331.2	31821.0

of 0.01 mM A2CA compared with the control plants. As shown in Table 1, free amino acid levels in the basal leaf regions (mentioned as "base") are higher than those in the differentiated parts (mentioned as "tip"). Data for contents of soluble proteins allow us to propose that amino acid accumulation is not due to protein hydrolysis.

It is well known that in bacteria as well as in higher plant cells resistance to amino acid analogues may be the result of overproduction of the related amino acid (Shiomi and Hori, 1973; Hasegawa, 1988). In our experiments we have detected in young leaf regions and in differentiated parts respectively about 5 and 20 times higher proline content in the regenerant A-5 than in the control plants. The regenerants A-1 and A-2 did not accumulate free proline either in the youngest leaf parts or in the mature ones.

Table 2. Effect of 0.01 mM A2CA on the intracellular content of some free amino acids

Regenerant	Leaf parts	Free amino acids (nmol.g^{-1} FW)						
		Ser	Lys	Tyr	His	Arg[1]	Val	Pro
Control	base	3341	101	102	438	341	306	610
	tip	4222	200	348	656	n.d.	292	301
A-1	base	15868*	988*	422*	1353*	2470*	1003*	511
	tip	6728	528	709	838	n.d.	926*	250
A-2	base	12459*	1034**	147	693	2819*	849	342
	tip	3981	327	586	565	n.d.	492	198
A-5	base	25472*	734*	273	1879*	1767*	1439*	3227*
	tip	9702	792*	270	1500	n.d.	660	6510**

* over 3-fold accumulation
** over 10-fold accumulation
[1] detected only in the youngest leaf parts
n.d. - not detected

As shown in Table 2, overproduction involved several other amino acids. The average values from two independent analyses of leaf extracts are expressed as nmol.g^{-1} fresh weight. Some accumulation of amino acids which are biosynthetically related to different metabolic pathways was evaluated. Our results are in agreement with those reported by Cella et al. (1982) and Mori et al. (1989) who detected elevated free serine and histidine content after selection for resistance to proline analogues in carrot cell lines and rice seedlings. Carbonera et al. (1989) reported that the addition of tryptophan to carrot cell cultures caused overproduction of intracellular free proline. An increase of contents of free lysine, histidine, alanine, leucine and phenylalanine was also estimated. The authors suppose that this phenomenon is probably due to exertion of a regulatory control by some amino acids on the biosynthesis of metabolically unrelated amino acids.

We demonstrate in this paper the possibility to obtain regenerants with altered amino acid pool from orchardgrass leaf explants cultured in the presence of proline analogues without any mutagenic treatment. A biochemical study on the mechanisms of resistance in the absence of proline accumulation could elucidate some properties of cellular amino acid metabolism.

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