

Isozymes as markers of the effect of growth regulator combinations on callus tissues from long-term cultures of *Cereus peruvianus* (Cactaceae)

Alberto José Prioli, Claudete Aparecida Mangolin, Sandra Aparecida de Oliveira and Maria de Fátima P.S. Machado

ABSTRACT

The maximum number of alcohol dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, acid phosphatase, peroxidase and esterase isozymes induced in the callus tissues from long-term cultures using three 2,4-dichlorophenoxyacetic acid (2,4-D) and N-(2-furanylmethyl)-1H-purin-6 amine (kinetin) combinations, was used as a criterion to evaluate the effect of growth regulators on callus tissue cultures of the cactus *Cereus peruvianus*. A total of 23 isozymes were induced in culture. Calli showing the largest number of induced isozymes were more abundant in the presence of higher auxin levels (2,4-D 4.0 mg/l x kinetin 4.0 mg/l). However among the six enzyme systems studied, only the number of alcohol dehydrogenase isozymes was correlated to 2,4-D and kinetin combinations. The number of isozymes induced by the different auxin-cytokinin proportions (2,4-D 4.0 mg/l x kinetin 4.0 mg/l, 6.0 mg/l or 8.0 mg/l) depended on the enzyme system considered, and none of the several isozymes induced could be used as markers of the effect of kinetin on shoot formation from callus tissues. A small proportion (10.6%) of regenerated *C. peruvianus* plants presented all 23 isozymes, indicating a transient expression of some of the induced isozymes.

INTRODUCTION

Growth regulators have an important effect on the developmental status of cells in plant tissue cultures (Meins, 1986). However little is known about how growth substances evoke a particular pattern of morphogenesis or the mechanism of induction at the cell and molecular levels (Thorpe, 1980). Changes in protein patterns have been used as specific markers to investigate such questions.

Direct evidence has shown that exogenous auxins stimulate the *de novo* synthesis of RNAs, and proteins in particular, in plant tissue cultures (Ricard *et*

al., 1976). Although there is no information on the mechanism that activates the synthesis of specific types of RNA or the possible functions of the proteins they synthesize, Reynolds (1989) demonstrated that during adventive organogenesis many preexisting proteins increase and some are newly synthesized from new translatable mRNA. He concluded that changes in gene expression occur during adventive organogenesis *in vitro*, most probably at the transcriptional level.

Cytokinins cause a slight increase in polysome level after subculture, but have no effect on the levels of a particular mRNA, or on the distribution of mRNAs between a non-translating and a translating pool, nor do they affect polysome level in the absence of subculture (Bevan and Northcote, 1981). Cytokinins

such as kinetin have been little exploited and understood in terms of their biological activity (Roberts and Hooley, 1988). However, when total proteins from calluses grown at two cytokinin concentrations were analyzed as molecular markers of cytokinin action the protein patterns varied significantly in their level of expression according to the treatment (Renaudim et al., 1991).

Since specific proteins can also be used as molecular markers of gene expression (Potter and Jones, 1991), we studied the relationship between changes in isozyme patterns which occurred in callus tissues from long-term culture, and different proportions of auxin and cytokinin in the culture medium.

MATERIAL AND METHODS

Since plants of the cactus *Cereus peruvianus* provide various compounds (a complex waxy lipid fraction, cellulose, and acidic gum used as an adjuvant in the flocculation of water impurities and in formulation of cosmetics) of economic, pharmacological and industrial interest (Alvarez et al., 1992), tissue-culture techniques have been developed for the rapid multiplication of this plant species (Oliveira, 1993). *C. peruvianus* plants were regenerated from calli cultured in medium containing three different combinations of the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and N-(2-furanylmethyl)-1H-purin-6 amine (kinetin). Isozyme patterns were determined by starch gel electrophoresis for alcohol dehydrogenase (ADH; EC 1.1.1.1), malate dehydrogenase (MDH; EC 1.1.1.37), isocitrate dehydrogenase (IDH; EC 1.1.1.42), acid phosphatase (ACP; EC 3.1.3.2), peroxidase (PER; EC 1.11.1.7), and esterase (EST; EC 3.1.1.1) and used as markers in the different tissues manipulated in tissue culture (Mangolin, 1993).

Eight hundred sixty nine callus tissues were cultured for two years in MS medium (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg et al., 1968) and the 2,4-D and kinetin combinations at concentrations of 4.0 mg/l 2,4-D and 4.0 mg/l, 6.0 mg/l, or 8.0 mg/l kinetin, and plants regenerated from calli were obtained and processed as reported by Machado et al. (1993). The starch gel electrophoresis conditions and the reaction mixtures for the visualization of the various isozymes of calli and regenerated plants were those reported by Mangolin (1993), Machado et al. (1993), and Mangolin et al. (1994a,b).

In the present study we demonstrated and evaluated the effect of these growth regulators in terms

of the maximum number of isozymes induced in the callus tissues cultured at each of the three 2,4-D and kinetin combinations used.

The contingency chi-square test (Steel and Torrie, 1980) was used to determine the probability association between the 2,4-D and kinetin combinations and the callus tissue showing the maximum number of isozymes induced in culture medium.

RESULTS

Two ADH (ADH-4, ADH-5) and MDH (mMDH-3, mMDH-5) isozymes, one IDH (IDH-2), six ACP (ACP_C-1, ACP_C-2, ACP_A-2, ACP_A-3, ACP_A-5, ACP_A-6), PER (PER_C-1, PER_C-3, PER_C-5, PER_A-1, PER_A-4, PER_A-5) and EST (EST_C-1, EST_C-2, EST_A-2, EST_A-3, EST_A-4, EST_A-5) isozymes were induced in callus tissues from long-term cultures of *C. peruvianus* at the following combinations of 2,4-D and kinetin: 4.0 x 4.0, 4.0 x 6.0 and 4.0 x 8.0 mg/l. Different numbers of callus tissues grown on the three 2,4-D and kinetin combinations showed the 23 isozymes induced in culture (Table I). The calli showing 11 (2 ADH, 2 MDH, 1 IDH, and 6 ACP) of the 23 isozymes were more abundant in the presence of higher auxin levels (2,4-D 4.0 mg/l x kinetin 4.0 mg/l). Callus tissues cultured for a long time at the three 2,4-D and kinetin combinations showed all the six PER and the six EST isozymes induced.

The number of callus tissues presenting only the two ADH isozymes was also greater when the 2,4-D 4.0 mg/l x kin 4.0 mg/l combination was used ($\chi^2 = 18.04$; $P < 0.01$). However, the number of callus tissues presenting either the two MDH or one IDH or the six ACP isozymes induced, was not correlated with the three 2,4-D and kinetin combinations (Table I).

The six PER (PER_C-5, PER_C-3, PER_C-1, PER_A-1, PER_A-4, PER_A-5) and six EST (EST_C-1, EST_C-2, EST_A-2, EST_A-3, EST_A-4, EST_A-5), ACP_C-2, ACP_A-2, ACP_A-3, ACP_A-5 isozymes induced were found at the same proportions in the callus tissues from long-term culture with the three 2,4-D and kinetin combinations while the other isozymes were found in different proportions of the callus tissues at the respective combinations 4.0 x 4.0, 4.0 x 6.0 and 4.0 x 8.0 mg/l of 2,4-D and kinetin (Table II).

The 23 isozymes induced in culture medium were observed in 10.6% of the plants regenerated from callus tissue. None of the 123 regenerated plants showed all the six ACP, PER and EST, or the two MDH isozymes induced in callus tissues from long-term culture. Only five and eight regenerated plants showed the

Table I - Test for independence between the 2,4-D and kinetin combinations (4.0 mg/l x 4.0, 6.0 or 8.0 mg/l) and the number of callus tissues from long-term cultures showing the maximum number of the ADH (2), MDH (2), IDH (1), ACP (6), PER (6), EST (6) isozymes induced in culture medium at the three 2,4-D and kinetin combinations.

Isozymes	2,4-D and kinetin combinations (mg/l)			χ^2 (contingency)	
	4 x 4 (N=350)	4 x 6 (N=307)	4 x 8 (N=212)		
	ADH	a	63		51
	b	66	67	34	
MDH	a	4	3	5	1.4 ^{NS}
	b	71	67	52	
IDH	a	11	13	8	1.72 ^{NS}
	b	20	19	17	
ACP	a	7	8	5	0.23 ^{NS}
	b	30	33	26	
PER	a	16	20	18	7.44*
EST	a	62	26	26	
Total	a	85	75	39	7.44*
	b	187	186	129	

*0.02 < P < 0.05; **P < 0.01; NS: Nonsignificant at the 5% level of probability; a: number of callus tissues showing the maximum number of the ADH (2), MDH (2), IDH (1), ACP (6), PER (6) and EST (6) isozymes induced in culture medium; b: number of callus tissues showing a variable number lower than the maximum number of isozymes induced in culture medium; χ^2 : test for independence.

two ADH isozymes and one IDH isozyme, respectively induced in the callus tissues from long-term cultures.

DISCUSSION

The largest number of isozymes was induced in the *C. peruvianus* callus tissues from long-term culture with the highest 2,4-D vs kinetin proportion (4.0 x 4.0 mg/l; Table I). Since the 2,4-D 4.0 mg/l and kinetin 8.0 mg/l combination was most effective for shoot regeneration (Oliveira, 1993) it seems that fewer isozymes are present in the regenerating callus (Man-

Table II - Proportions of the callus tissues from long-term culture in the three 2,4-D and kinetin combinations (4.0 x 4.0, 4.0 x 6.0 and 4.0 x 8.0 mg/l) showing the 23 isozymes induced in the culture medium.

Induced isozymes	2,4-D kinetin combinations (mg/l)		
	4.0 x 4.0	4.0 x 6.0	4.0 x 8.0
ADH-4	0.9848	0.8358	0.7941
ADH-5	0.9545	0.7611	0.6176
MDH _m -3	0.4788	0.2985	0.5192
MDH _m -5	0.0563	0.0447	0.0961
IDH-2	0.5500	0.6842	0.4705
ACP _a -1	0.2333	0.2424	0.1923
ACP _a -2	1.0000	1.0000	1.0000
ACP _a -3	1.0000	1.0000	1.0000
ACP _a -4	0.7000	0.7272	0.7307
ACP _a -5	1.0000	1.0000	1.0000
ACP _c -2	1.0000	1.0000	1.0000
PER _a -1	1.0000	1.0000	1.0000
PER _a -4	1.0000	1.0000	1.0000
PER _a -5	1.0000	1.0000	1.0000
PER _c -1	1.0000	1.0000	1.0000
PER _c -3	1.0000	1.0000	1.0000
PER _c -5	1.0000	1.0000	1.0000
EST _a -2	1.0000	1.0000	1.0000
EST _a -3	1.0000	1.0000	1.0000
EST _a -4	1.0000	1.0000	1.0000
EST _a -5	1.0000	1.0000	1.0000
EST _c -1	1.0000	1.0000	1.0000
EST _c -2	1.0000	1.0000	1.0000

golin *et al.*, 1994a). The total amount of protein in a regenerating callus of a *Petunia* species was also lower than in unregenerating callus (Renaudin *et al.*, 1991).

However, a careful analysis of each enzyme system in callus tissues of *C. peruvianus* showed that the lower number of isozymes present in the regenerating callus is not a generalized event. Only the number of ADH isozymes of the six enzyme systems studied was related to the 2,4-D and kinetin combinations. Furthermore, a higher number of calli showing the six PER and the six ACP isozymes was observed at the 2,4-D 4.0 mg/l and kinetin 6.0 mg/l combination, and the same number of callus tissues presenting the six EST isozymes was detected in the 2,4-D 4.0 mg/l x kinetin 6.0 mg/l and 2,4-D 4.0 mg/l x kinetin 8.0 mg/l combinations.

Therefore, the larger or lower number of isozymes induced by the different auxin/cytokinin proportions depends on the enzyme system considered.

Thus, the transcription protein factors induced by the action of auxins at the molecular level proposed by Ricard *et al.* (1976), which lead to the transcription of particular proteins, probably have a mode of action which depend on the auxin/cytokinin ratio and specific enzymes as well as on the plant species. This because, contrary to what is observed in the massive synthesis of peroxidases in lentil root tissue under the effect of auxin (Ricard *et al.*, 1976), we observed that the higher 2,4-D/kinetin proportion did not induce the largest number of isoperoxidases in callus tissues from long-term cultures of *C. peruvianus*. In *C. peruvianus*, the ADH isozymes were the specific proteins having the highest sensitivity as molecular markers related to the changes occurring in callus tissues from long-term culture, showing the greatest reaction at the highest auxin/cytokinin ratio.

Although the appearance of specific proteins has been correlated with organogenesis treatments (Reynolds, 1990), none of the isozymes induced in the *C. peruvianus* calli were exclusively found at a particular 2,4-D and kinetin combination. The several induced isozymes were found in different proportions of the callus tissues from long-term culture with the various 2,4-D and kinetin combinations (Mangolin *et al.*, 1994b). But these different proportions were not accompanied by an increase of the kinetin level, or by a specific kinetin/2,4-D ratio. Thus, the effect of kinetin level on plant shoot formation (Krikorian *et al.*, 1988) as well as on *C. peruvianus* shoot regeneration at the 4.0 x 8.0 mg/l combination was not correlated with any of the specific isozymes induced.

Several of the isozymes induced in callus tissues of the *C. peruvianus* species could be used as markers for plants regenerated from callus tissues (Mangolin, 1993; Machado *et al.*, 1993). However, only the ADH isozymes can be used as markers for the different 2,4-D and kinetin combinations in callus tissue from long-term culture (Mangolin *et al.*, 1994a).

The low proportion of the *C. peruvianus* regenerated plants showing all the 23 isozymes induced in callus tissues from long-term culture indicates a transient expression of some of the induced isozymes.

Therefore, the study of isozymes in callus tissues from long-term culture of *C. peruvianus* supports the evidence of an effect of auxins on the induction of particular polypeptides, but shows that this effect is solely dependent on the growth regulator combinations for a specific enzyme system such as the ADH isozymes studied here. The number of ADH isozymes induced depended on the 2,4-D and kinetin concentrations, but none of the specific ADH or the MDH, IDH, ACP, PER and EST isozymes induced in this study act as direct

markers of an effect of kinetin on shoot formation from callus tissues of *C. peruvianus* cactus cultured for two years.

ACKNOWLEDGMENTS

This work was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

RESUMO

O número máximo de isozimas álcool desidrogenase, malato desidrogenase, isocitrato desidrogenase, peroxidase e esterase, induzidas em tecidos de calos cultivados por longo tempo em três combinações de ácido 2,4-diclorofenoxiacético (2,4-D) e cinetina (kin), foi usado para demonstrar e avaliar o efeito de reguladores de crescimento em cultura de tecidos de calos do cactus *Cereus peruvianus*. Foram induzidas um total de vinte e três isozimas em cultura. Calos com maior número de isozimas induzidas foram mais abundantes na presença de maior nível de auxina (2,4-D 4.0 mg/l x kin 4.0 mg/l). Entretanto, dos seis sistemas enzimáticos estudados, somente o número de isozimas álcool desidrogenase apresentou associação com as combinações de 2,4-D e kin. O maior ou menor número de isozimas induzidas nas diferentes proporções de auxina-citocinina (2,4-D 4.0 mg/l x kin 4.0 mg/l, 6.0 mg/l ou 8.0 mg/l), foi dependente do sistema enzimático considerado, e nenhuma das diversas isozimas induzidas puderam ser usadas como marcadores do efeito de cinetina na formação de caules a partir de tecidos de calos. Uma baixa proporção (10,6%) de plantas regeneradas apresentando todas as vinte e três isozimas, indica uma expressão transitória de algumas das isozimas induzidas em cultura nos tecidos de calos de *C. peruvianus*.

REFERENCES

- Alvarez, M., Costa, S.C., Utumi, H., Huber, A., Beck, R. and Fontana, J.D. (1992). The anionic glycan from the cactus *Cereus peruvianus*. *Appl. Biochem. and Biotechnol.* 34/35: 283-295.
- Bevan, M. and Northcote, D.H. (1981). Subculture-induced protein synthesis in tissue cultures of *Glycine max* and *Phaseolus vulgaris*. *Planta* 152: 24-35.
- Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.
- Krikorian, A.D., Kelly, K. and Smith, D.L. (1988). Hormones in tissue culture and micropropagation. In: *Plant Hormones and their Role in Plant Growth and Development* (Davis, P.J., ed.). Kluwer Academic Publishers, Dordrecht, pp. 593-613.

- Machado, M.F.P.S., Prioli, A.J. and Mangolin, C.A.** (1993). Malate dehydrogenase (MDH; EC 1.1.1.37) isozymes in tissue and callus cultures of *Cereus peruvianus* (Cactaceae). *Biochem. Genet.* 31: 167-172.
- Mangolin, C.A.** (1993). Isoenzimas em cultura de tecidos de *Cereus peruvianus* (Cactaceae). Master's Thesis, Universidade Estadual de Maringá, PR.
- Mangolin, C.A., Prioli, A.J. and Machado, M.F.P.S.** (1994a). Alcohol dehydrogenase (ADH; EC 1.1.1.1) isozymes as markers of 2,4-D x Kinetin combinations in callus cultures of *Cereus peruvianus* (Cactaceae). *Biochem. Genet.* 32: 191-200.
- Mangolin, C.A., Prioli, A.J. and Machado, M.F.P.S.** (1994b). Isozyme patterns in callus cultures and in plants regenerated from calli of *Cereus peruvianus* (Cactaceae). *Biochem. Genet.* 32: 237-247.
- Meins, F. Jr.** (1986). Determination and morphogenetic competence in plant tissue culture. In: *Plant Cell Culture Technology* (Yeoman, M.M., ed.). Blackwell Scientific Publications, Oxford, pp. 7-25.
- Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue. *Physiol. Plant.* 15: 474-497.
- Oliveira, S.A.** (1993). Cultura de tecidos e regeneração de plantas de *Cereus peruvianus* (Cactaceae). Master's Thesis, Universidade Estadual de Maringá, PR.
- Potter, R.H. and Jones, M.G.K.** (1991). Molecular analysis of genetic stability. In: *In Vitro Methods for Conservation of Plant Genetic Resources* (Doods, J.H., ed.). Chapman and Hall, London, pp. 71-91.
- Renaudin, J.P., Tournaire, C. and De la Serve, B.J.** (1991). Quantitative analysis of protein changes during meristem initiation and bud development in protoplast-derived *Petunia hybrida* callus. *Physiol. Plant.* 82: 48-56.
- Reynolds, T.S.** (1989). Change in RNA, protein and translatable messenger RNA synthesis and accumulation during adventive organogenesis in somatic tissue cultures of *Solanum carolinense*. *Plant Science* 65: 77-85.
- Reynolds, T.S.** (1990). A two-dimensional electrophoresis analysis of protein synthesis and accumulation during adventitious shoot formation in somatic tissue cultures of *Solanum carolinense* L. *J. Plant Physiol.* 136: 213-218.
- Ricard, J., Teissere, M., Azou, Y. and Penon, P.** (1976). Hormonal control of ribonucleic acid and protein synthesis in plants. *J. Microscopie Biol. Cell* 26: 139-150.
- Roberts, J.A. and Hooley, R.** (1988). Receptors - sites of perception or deception? In: *Plant Growth Regulators* (Roberts, J.A. and Hooley, R., eds.). Blackie and Son Limited, New York, pp. 134-150.
- Steel, R.G.D. and Torrie, J.H.** (1980). *Principles and Procedures of Statistics: A Biometrical Approach*. 2nd. edn. McGraw-Hill, New York, pp. 633.
- Thorpe, T.A.** (1980). Organogenesis *in vitro*: structural, physiological, and biochemical aspects. *Int. Rev. Cytol. Suppl.* 11A: 71-111.

(Received January 3, 1994)