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# Esterase activity and isoenzymes in relation to morphogenesis in *Mammillaria gracillis* Pfeiff. tissue culture

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Cactus *Mammillaria gracillis* Pfeiff. (*Cactaceae*), cultivated *in vitro*, spontaneously switches from an organised to unorganised way of growth, producing a habituated organogenic callus which regenerates normal and hyperhydric shoots without the addition of any growth regulators. Tumour tissues, induced by *A. tumefaciens* wild strain B6S3 (tumour TW) and rooty mutant GV3101 (tumour TR), do not express any organogenic potential. The esterase (arylesterase EC 3.1.1.2 and carboxylesterase EC 3.1.1.1) activity and isoenzyme pattern of morphologically different cactus tissues: shoot, callus, hyperhydric regenerant, tumours TW and TR, were compared. Tissue samples were frozen at –80 °C and lyophilized before protein extraction. Two esterase substrates, 1- and 2-naphthylacetate, were used. Esterase activity of all tissues varied during the period of one subculture. In shoots and tumours, the highest esterase activity for both substrates was measured on the 21$^{st}$ day, while in the callus and hyperhydric regenerants the highest activity was on the 7$^{th}$ day. Esterases were separated electrophoretically in polyacrylamide gradient gels under non-denaturating conditions. In total, 13 isoesterases, reacting with both substrates, were resolved. No differences in isoenzyme profile were noticed in correlation with the age of tissues, but the esterase activity varied among tissues. The significance of these results is discussed.

**Key words:** Cactus, tumour, crown-gall tumour, callus, habituated callus, hyperhydric regenerants, isoesterase, *Mammillaria gracillis*, morphogenesis

*Abbreviations:* BSA – bovine serum albumin; 2-D PAGE – two-dimensional polyacrylamide gel electrophoresis; PVP – polyvinylpyrrolidone; SDS – sodium dodecyl sulphate.

## Introduction

Numerous enzymes were tested as markers of developmental processes in plants, but there are few reports related to cacti species (TORQUATO et al. 1995, JORGE et al. 1997,

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MANGOLIN et al. 1997). As xerophytes with Crassulacean Acid Metabolism (CAM), cacti are highly affected by the artificial environmental conditions in tissue culture (MALDA et al. 1999). *In vitro* propagated plants of *Mammillaria gracillis* Pfeiff., under hyperhydric conditions in the culture, develop calluses without any exogenous growth regulators (KRSNIK-RASOL and BALEN 2001). This system, in which a cactus with a characteristic organisation pattern easily turns to unorganised growth, is convenient for plant development studies (BALEN et al. 2002, POLJUHA et al. 2003, BALEN et al. 2003a). A habituated callus can be compared with a crown-gall tumour, which is also hormone-independent; therefore, crown-gall tumours were induced by *Agrobacterium tumefaciens* wild strain B6S3 (tumour TW) and rooty mutant GV3101 (tumour TR).

Enzymes such as esterases, which hydrolyse ester bonds, generally have a broad spectrum of substrates and act on a wide variety of natural and xenobiotic compounds. One- and 2-naphthylacetate are often used for visualisation of these enzymes in plant tissues (PEŠKAN et al. 1996, KRSNIK-RASOL et al. 1999, CUMMINS et al. 2001, BALEN et al. 2003a). These compounds are substrates of arylesterases (EC 3.1.1.2), which also hydrolyse organophosphorous acid esters, and of carboxylesterases (EC 3.1.1.1), which are inhibited by some organophosphates. Esterase isoform polymorphism of *Mammillaria* tissues was previously studied by using 1- and 2-naphthylacetate as substrates (BALEN et al. 2003a). Esterases were more suitable biochemical markers of developmental processes in the *Mammillaria gracillis* tissue culture than peroxidases, and had a greater number of expressed isoforms. The morphogenic statuses of *Mammillaria* tissues and esterase activity are related. The isoesterase patterns differ among the organised (shoots and normal regenerants) and unorganised (callus and hyperhydric regenerants) untransformed tissues. TW and TR tumours differ from untransformed tissues but also from each other with respect to esterase isoenzyme patterns.

The abundant polysaccharides present in *Mammillaria* species make the extracts viscous and difficult to manipulate. Since the esterases appeared to be good markers of plant morphogenesis, we tried to improve the method for the separation of esterases related to morphogenesis in *Mammillaria gracillis* tissue culture. In this paper we report modifications in the preparation of tissue extracts in order to achieve more reproducible results as well as the analysis of the enzyme activity and isoenzyme pattern in correlation to the age of tissues.

## Materials and methods

### Plant material

The following plant material was studied: *Mammillaria gracillis* Pfeiff. shoots, habituated calluses, hyperhydric regenerants and two tumour lines. These tissues were cultivated *in vitro* on the solid, hormone-free MS (MURASHIGE and SKOOG 1962) nutrient medium (0.9 % agar, 3 % sucrose) under 16/8-hour-light/night photoperoid (light intensity 90 $\mu E\ s^{-1}\ m^{-2}$) at 24° C. In the culture conditions, without exogenous growth regulators an abundant callus was formed at the basis of cactus plants (KRSNIK-RASOL and BALEN 2001). This callus, subcultivated on hormone-free MS medium, expressed a high morphogenic capacity, simultaneously regenerating phenotypically normal and malformed hyperhydric shoots. Tumour lines were established from primary tumours induced on shoot explants by *Agrobacterium tumefaciens*, the wild strain B6S3 (tumour line TW) and rooty mutant

GV3101 (tumour line TR) (KRSNIK-RASOL and BALEN 2001). Transformed tissues never expressed any morphogenic potential.

### Enzyme extraction and protein quantification

For enzyme extractions different *Mammillaria* tissues were collected every 7 days during the period of one subculture (28 days). Tissue samples were frozen at –80 °C and lyophilized before protein extraction. Protein extracts of tissues of different ages were prepared at the same time and under the same conditions. For measurements of esterase activity and electrophoretic isoenzymes analysis extracts were prepared in an ice-cold 0.1 M Tris/HCl buffer, pH 8.0 (STAPLES and STAHMAN 1964), with the addition of PVP. Per 1.0 g of fresh tissue, 1.5 mL of buffer was used. Homogenates were centrifuged 15 min at 20 000 × *g* and 4 °C. Supernatants were centrifuged again for 60 min under the same conditions. The protein content of supernatants (crude extracts) was determined according to BRADFORD (1976) using BSA (Sigma Chemical Co, New York) as a standard. Experiments were repeated four times.

### Enzyme assays

For esterase activity measurement, 1- and 2-naphthylacetate (Sigma Chemical Co, New York) were used as broad spectrum substrates for esterases (arylesterase and carboxylesterase) according to BURLINA and GALZIGNA (1972). The esterase activity was determined spectrophotometrically at room temperature (23 °C) by measuring the increase in absorbance at 322 nm (for 1- naphthylacetate) and 313 nm (for 2- naphthylacetate). The reaction solution contained 1.5 ml 0.1 M Tris/HCl pH 7.4 and 30 μL 100 mM 1-naphthylacetate or 2-naphthylacetate dissolved in absolute methanol. For each measurement 200 μL of crude extract was used. Measurements were performed in 1.0 cm cuvettes every 15 seconds over a three-min-period. The esterase activities were corrected for spontaneous hydrolysis of 1- and 2- naphthylacetate. The activities were calculated using the molar absorption coefficients of $\varepsilon_{1\text{-naphthol (322 nm)}} = 2 \times 10^3\ M^{-1}\ cm^{-1}$ and $\varepsilon_{2\text{-naphthol (313 nm)}} = 1.25 \times 10^3\ M^{-1}\ cm^{-1}$ (BALEN et al. 2003a). The absorption coefficients for 1- and 2-naphthol were corrected for the absorption coefficients of the acetate esters measured at the same wavelengths. The blank contained the buffer and corresponding naphthylacetate. The activity was expressed as μmol of hydrolysed substrate per min and per g of fresh weight (μmol $min^{-1}\ g^{-1}$). Four series of tissue samples were assayed for esterase activity.

### Statistical analysis

The results were given as means (4 replicates of each tissue) ± standard errors and compared by one-way ANOVA followed by Duncan's New Multiple Range Test (DUNCAN 1995).

### Electrophoretical separation of isoenzymes

Tissue extracts were analysed electrophoretically under non-denaturating conditions using vertical polyacrylamide, gradient 8–18% (w/v) slab gels with the buffer system of LAEMMLI (1970). A constant voltage of 200 V was applied for 5 h and the temperature maintained at 4 °C. Equal amounts of protein were loaded onto each lane.

### Enzyme activity staining

For visualisation of isoesterases the following solution was prepared. Forty mg of 1-naphthylacetate and 40 mg of 2-naphthylacetate were dissolved, together and also separately, in 16 ml of 50% (v/v) acetone and mixed with 100 ml of 50 mM Tris/HCl buffer (pH 7.1). The gels were incubated for 30 min in this solutions, rinsed in tap water, and stained 10 to 20 min in 0.2 % Fast Blue RR salt (Sigma Chemical Co, New York) solution. The Fast Blue RR salt was dissolved in an appropriate volume of absolute methanol, and filtered into 50 mM Tris/HCl buffer (pH 7.1) (CHELIAK and PITEL 1985). The gel was rinsed in tap water and fixed in 30% (v/v) ethanol.

## Results

Morphologically different *Mammillaria* tissues were compared with regard to esterase activity for two substrates: 1- and 2-naphthylacetate. Changes in enzyme activity related to tissue aging during one subculture (28 days) were also followed.

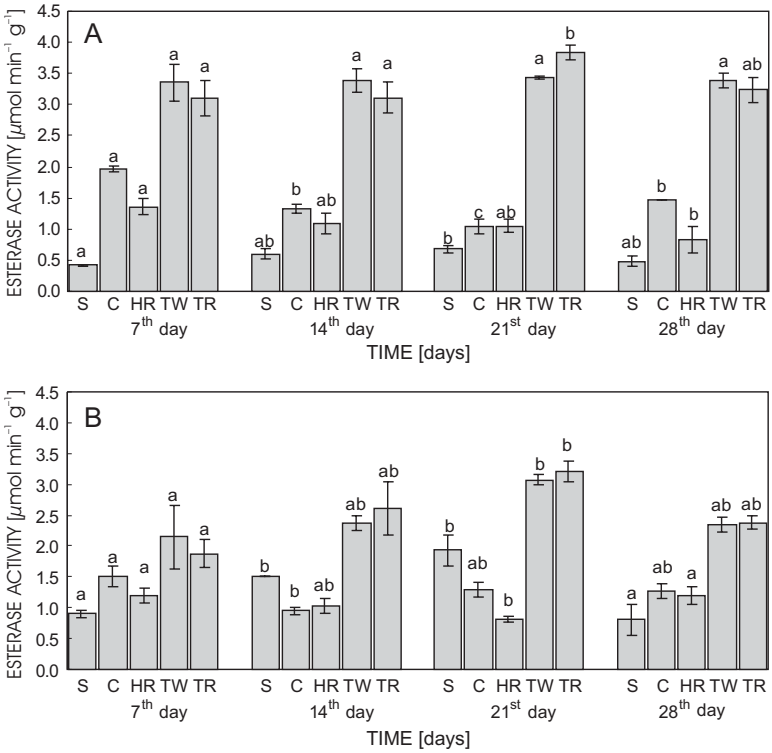


**Fig. 1.** Esterase activity during one subculture (28 days) with 1-naphthylacetate (A) and 2-naphthylacetate (B) as substrates in *Mammillaria gracillis* tissue lines (± SE). Extracts of the same tissue sample of different age were compared according to Duncan's New Multiple Range Test. Significantly different values ($P < 0.05$) are marked with different letters (a, b, c). S-cactus shoot, C-habituated callus, HR-hyperhydric regenerant, TW-tumour TW, TR-tumour TR.

When 1-naphthylacetate was used as a substrate (Fig.1A), the lowest esterase activity was measured in shoot extracts and the highest in tumour tissue. Calluses and hyperhydric regenerants had higher activity than shoots but lower than tumours. Except on the 21st day, calluses had higher activity than hyperhydric regenerants produced by calluses. In the TW tumour, no significant difference in esterase activity was observed during the subculture, although the highest esterase activity was measured on the 21st day. The TR tumour had the highest activity on the 21st day.

When 2-naphthylacetate was used as a substrate (Fig 1B), shoot extracts had higher activity than calluses and hyperhydric regenerants on the 14th and 21st day. The highest esterase activity in calluses and hyperhydric regenerants was measured on the 7th day. The esterase activity of both tumours increased with the growth of tissues until the 21st day when it reached the highest value. In shoots and tumours, the highest esterase activity for both substrates was measured on the 21st day, while in the calluses and hyperhydric regenerants it had the highest value on the 7th day.

In total, 13 (E1 – E13) isoesterases, reacting with both substrates together, were resolved electrophoretically (Fig. 2). The majority of the bands were common to all tissue extracts. Although the isoforms E1 and E2 were present in all samples, they were more pronounced in extracts of the TW and TR tumours. The pink coloured bands E4 and E9 were characteristic of hyperhydric regenerants and calluses, while the faint band E8 was missing in extracts of both tumours. Isoesterase E10 was absent in shoot samples and had the strongest coloration in TW tumour. The pink brown isoenzyme E11 was the most intensively coloured band in all tissue extracts. Very faint bands, E12 and E13, were present in all samples, except in the TR tumour. No differences in isoenzyme profile were noticed in correlation to the age of tissues, except for fainting of the band E10 in extracts of calluses, hyperhydric regenerant and TR tumour.

When gels were treated with each substrate separately, 1-naphthylacetate gave a dark brown, while the 2-naphthylacetate gave a pinky brown coloration. It was shown that all 13 esterase isoforms reacted with both substrates (Fig. 3A and 3B).

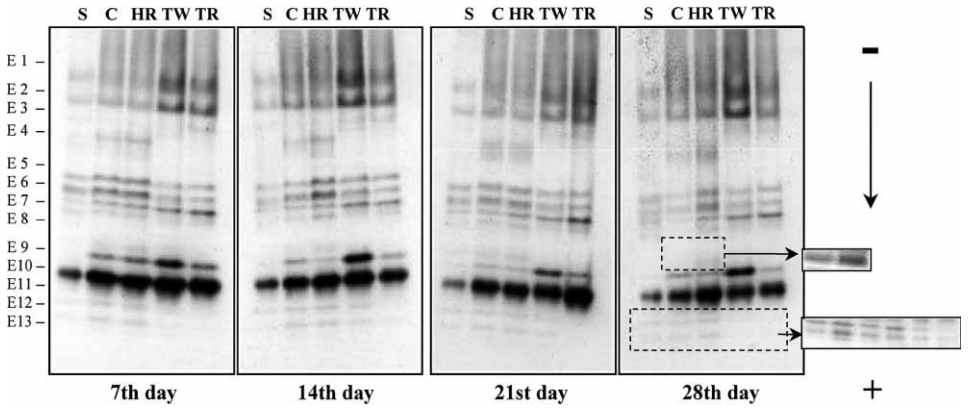


**Fig. 2.** Isoesterase patterns of *Mammillaria gracillis* tissue lines during the period of one subculture (7th, 14th, 21st and 28th day are marked below). Gels were incubated in a solution of both substrates together. S-cactus shoot, C-habituated callus, HR-hyperhydric regenerant, TW-tumour TW, TR-tumour TR. 50 µg of proteins were loaded onto each gel lane.

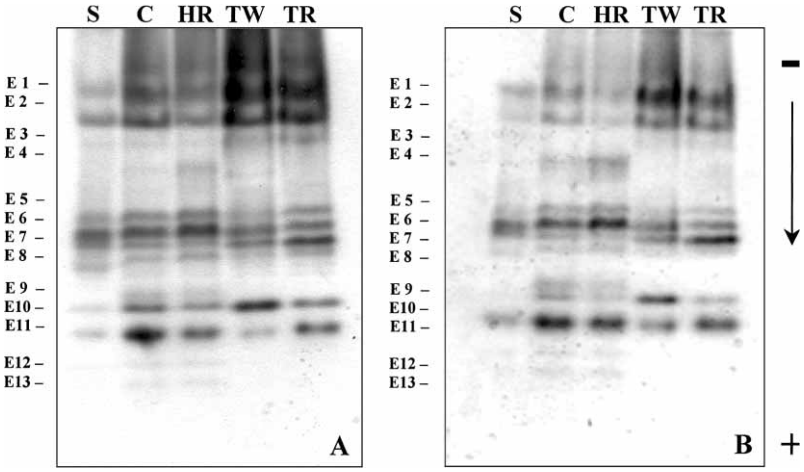


**Fig. 3.** Isoesterase pattern of *Mammillaria gracillis* tissue lines. Gels were incubated with 1-naphthylacetate (A) or with 2-naphthylacetate (B). S-cactus shoot, C-habituated callus, HR-hyperhydric regenerant, TW-tumour TW, TR-tumour TR. 50 μg of proteins were loaded onto each gel lane.

## Discussion

In a previous study of the same authors cactus shoots, calluses, normal and hyperhydric regenerants and both tumours were compared with regard to esterase activity and isoenzyme pattern (BALEN et al. 2003a). It was concluded that, in *Mammillaria gracillis* system, esterases were suitable biochemical markers of developmental processes. The esterase activity was increasing with the decrease of the tissue organisation level and all undifferentiated tissues (calluses and both tumours) had significantly higher activity than shoots and regenerants, with both substrates. The isoenzyme pattern was complex and related to the certain morphological stage. COPPENS and DEWITTE (1990) used esterase zymograms from barley callus as a biochemical marker of embryogenesis and organogenesis and concluded that esterase system was very sensible for detection of embryogenesis before somatic embryos are formed. ABRAMS (1976) reported that morphological level of tissue as well as culture conditions in sunflower and tobacco tissue culture influenced esterase expression. In potato tuber tissue change in isoesterase pattern was observed during tumour development while distinct isoesterase patterns were noticed in sugar beet normal, habituated and crown gall tumour tissues (KRSNIK-RASOL et al. 1999).

In the present study some modifications of the sample preparation were introduced. Extract preparation of cactus tissue (especially shoots and regenerants) was very difficult due to tissue toughness and the presence of abundant secondary metabolites, which were indicated by the viscous, glue-like texture of extracts. The presence of polysaccharides was already reported for DNA extracts of two cacti genera, *Hylocereus* and *Selenicereus* (TEL--ZUR et al. 1999). Homogenisation of fresh shoot tissue was not satisfactory and the loss of tissue that could not be homogenised was substantial. Therefore, the protein concentration of these extracts was low and the results of electrophoretic analysis were sometimes irreproducible. After lyophilization of all tissues before protein extraction, the measurement of

esterase activity was more accurate and isoenzyme pattern analysis gave more reproducible profiles then in previous experiments (BALEN et al. 2003a). Tissue extracts were prepared in the Tris/HCl buffer, pH 8.0, while in the previous study (BALEN et al. 2003a) samples were prepared in the phosphate buffer, pH 7.0. The enzyme activity and isoenzyme profiles were not significantly affected by change of composition or pH value of the extraction buffer. The guaiacol peroxidase activities of the samples prepared in Tris/HCl buffer pH 8.0 was approximately five times lower than the activities of samples prepared in the phosphate buffer, pH 7.0 (BALEN *et al*. 2003b).

Changes in enzyme activity as well as in isoenzyme profile were followed during one subculture (28 days) in order to establish changes related to tissue aging in *Mammillaria gracillis* tissue culture. Although no changes in isoenzyme profile were noticed, the esterase activity varied among tissues of different ages. This result can be explained by higher sensitivity of calluses and hyperhydric regenerants; degradation and necrosis of these tissues appear earlier than in tumour and shoot tissue. Time course studies of the *Fritillaria puqiensis* peroxidase and esterase during the bulb organogenesis *in vitro* revealed that different isoenzymes are associated with organogenesis and express activity at different times (CAI et al. 1999). MARTINELLI and GIANAZZA (1996) investigated the biochemical changes during regeneration of sunflower and reported that the isoesterase pattern was related to the genotype as well as to the morphogenic phase.

The isoesterase patterns were identical when 1- and 2-naphthylacetate were used separately. When gels were treated with both substrates together, some bands were dark brown while the others were pinky brown (BALEN et al. 2003a). It was difficult to conclude whether each isoform reacted with both substrates. Similar observations on the colour of isoesterase bands were reported by GRIFFITH and BANOWETZ (1992) for zymograms of *Lolium* seed extracts. In comparison to our previous study (BALEN et al. 2003a) some new or more pronounced bands were noticed. Previously the isoesterase E3 was present only as a faint band in shoots and TR tumour, while in this study it was visible in all extracts. The new bands E8 and E9, which did not appear before, were noticed in untransformed tissues and calluses and hyperhdric regenerants, respectively. All 13 detected isoesterases were noticed in the extracts of calluses and hyperhydric regenerants, while 10 of them were present in cactus shoots. The TW and TR tumours were different from untransformed tissues as well as among each other with respect to the isoesterase profiles.

Normal shoots, regenerated from habituated callus, were omitted from this analysis. Previously it was shown that the normal regenerants differ from shoots (BALEN et al. 2003a). Esterase activity of normal regenerants was only slightly higher than in shoot extracts, while the isoenzyme pattern was identical.

## Conclusion

Tissue extracts prepared in the Tris/HCl buffer, pH 8.0 gave similar enzyme activities and isoenzyme profiles as extracts prepared in the phosphate buffer, pH 7.0. The reproducibility of esterase activities and electrophoretic profiles of extracts prepared in the Tris/HCl buffer, pH 8.0 was very good and these extracts can also be used for several other protein analyses (SDS-PAGE, 2-D PAGE, etc). Esterase activity of all tissues varied during the period of one subculture, while no differences in isoenzyme profile were noticed in correla-

tion to the age of tissue. The isoesterase patterns were identical when substrates were used separately. All 13 isoesterases were present in the extracts of callus and hyperhydric regenerants. It was confirmed that the morphogenic status of different *Mammillaria* tissues was reflected in esterase activity and its isoenzyme pattern.

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