Transglutaminase activity and localization during microspore induction in maize

M. Santos[1], J. Alché[2] , M.I. Rodríguez-García[2] and J.M.Torné[1]

[1] Department of Molecular Genetics, Laboratori de Genetica Molecular Vegetal, Consorci CSIC-IRTA. IBMB. Jordi Girona 18-26. 08034 Barcelona, Spain.
[2]Department of Biochemistry, Cell and Molecular Biology of Plants. Estación Experimental del Zaidín, CSIC. Profesor Albareda 1. 18008 Granada, Spain.

As discussed in the previous chapter, the functionality of plant transglutaminases (TGases) has not been studied to any great extent. To delve more into the role of this enzyme in the plant cell, both the enzyme activity and the polyamine content during different stages of *in vivo* and *in vitro* development of the maize pollen grain have been analysed. A preliminary study on TGase immunolocalization in maize (*Zea mays*) pollen and its relationship with the process of *in vitro* androgenesis has also been carried out. Enhanced TGase activity and higher levels of polyamine content were observed in induced microspores in comparison with non-induced microspores and mature pollen grains, the latter exhibiting lower levels of both enzyme activity and polyamines. The signal observed after TGase immunolocalization by FM coincided with these data. Ultrastructural localization of TGase showed that the enzyme was associated with membrane systems and in poorly-differentiated organelles. The results indicate a putative role for TGase in the changes during androgenesis induction, most likely catalysing cross linking between polyamines and proteins related to the formation of pollen membrane systems.

Keywords: maize, pollen, microspores, androgenesis, transglutaminase

1. Introduction

Pollen formation is the result of finely coordinated biochemical, physiological, cytological and morphological events that normally take place in the anthers of developing inflorescences. There is little information concerning the physiology and biochemistry of pollen development and maturation in cereals. Male and female inflorescences of maize have been cultured to study *in vitro* gametophyte development, and to develop and produce mature pollen capable of germination for application in crop improvement [1]. The *in vitro* culture of intact anthers containing haploid microspores has been used to obtain regenerable maize tissue cultures that could be very useful as target cells in experiments aimed at genetic transformation and breeding [2]. In this system, uninucleate microspores are induced to undergo androgenesis (by cold pre-treatment) when still within the anther, resulting in haploid embryos capable of plant regeneration [3], but the frequency of androgenesis has been relatively low. In the last few years, cultivars with high levels of androgenesis response have become available, as well as procedures for high frequency regeneration of plants from isolated microspore cultures of maize [4]. Nevertheless, these results have been obtained in a reduced number of genotypes and biochemical and molecular factors involved have been insufficiently analyzed.

The polyamines diamine putrescine (Put) and the polyamines spermidine (Spd) and spermine (Spm) are involved in numerous processes of plant growth and development [5]. Floral initiation seems to be related to a high Spd concentration. Conjugates of these amines with hydroxycinnamic acids have been found in reproductive organs such as anthers, pollen, ovaries and seeds, and are absent in male-sterile maize cultivars. High Spd titers have also been observed in many embryogenic cultures. [6] [7] [8].

Transglutaminase (TGase) activity has been detected in the pollen of *Malus domestica* [9] where tubuline seems to be one of its preferential substrates. It is also well known that this enzyme catalyzes cross linking between polyamines and proteins in the plant cell and, consequently, it may be involved in cell-

differentiation processes. We have published research on the involvement of polyamine in pollen maturation *in vivo* and androgenesis induction *in vitro* in a maize genotype [10] [11], and here present an introduction to the study on TGase involvement in these processes.

Enzyme activity and polyamine (PA) content in immature microspores (induced and non-induced) and mature maize pollen have been analysed. In parallel, the immunolocalization of the enzyme in the three different stages of pollen differentiation by western blot analyses and by FM and MET is presented.

In order to illustrate the results described later, the different stages of *in vitro* maize pollen differentiation are presented in Figure 1.

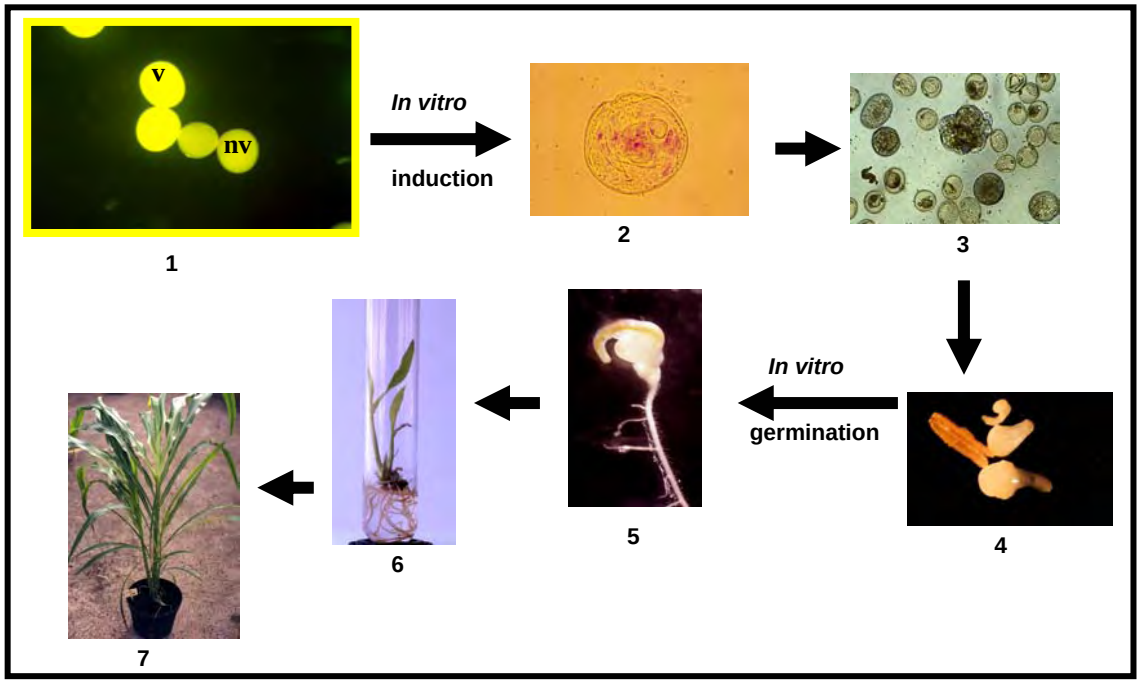

Figure 1- *In vitro* androgenesis induction and plant regeneration from immature maize microspores. 1, viable (v) and non-viable (nv) immature pollen (fluorescein diacetate staining) ; 2, 15-day cultured multinucleate pollen (acetocarmine staining), 3, 30-day cultured pollen at different stages of *in vitro* microspore development (see magnification in Figure 1a), 4, 40-day cultured androgenetic embryo; 5, *in vitro* embryo germination; 6, *in vitro* plant differentiation, 7, normal regenerating maize plant (from Santos et al. 1995; Willadino et al. 1995).

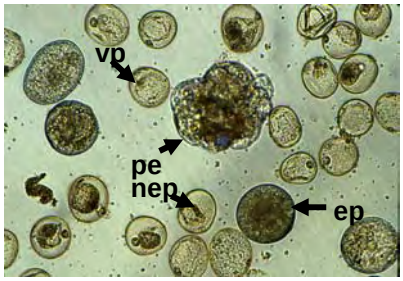

Figure 1a- *In vitro* pollen suspension (inverted microscope)
vp, vacuolated pollen; ep, embryogenic pollen; npe, non-embryogenic pollen, pe, pro-embryoid

2. Polyamine content and transglutaminase activity

The endogenous content of free and bound polyamines was previously analyzed over 30 days of pollen evolution, in both developmental pathways (androgenesis and microsporogenesis) [10]. As shown in Figure 1, the induction of androgenesis from cold pre-treated uninucleate pollen (10 days at 10ºC) resulted, in most cases, in a different pattern of Put and Spd content with respect to the normal pollen developmental pathway (microsporegenesis). These differences indicate that PA metabolism is altered during the induction of androgenesis. In general, pollen developmental stages that involve cell division (tetrades, pre-anthesis pollen and four-day cultured pollen) are characterized by a predominance of free Spd (Figure 2 C and D). The increase of Spd in 15-day cultured pollen, when the first embryoids are formed, indicate the possible implication of these PAs in embryogenesis. However, bound Put content was, in general, predominant with respect to free Put in both processes, being higher in the 15-day cultured pollen and in uninucleate pollen (Figure 1 A and B). Since bound PAs are predominantly formed by cross linking to proteins via TGase activity [12], these latter results might be related to the TGase activity detected in the different stages of pollen development (Figure 3). The highest enzyme activity is detected in the cold-induced uninucleated microspores (IP) (probably due to the stress produced in the cells), the activity is lower in uninucleate (U) and germinated pollen (GP), and mature pollen (MP) has very low activity. The different PA content observed in our maize androgenetic pollen with respect to the *in vivo* maize pollen may also be due to the involvement of polyamines in morphogenetic processes, in which TGase is also involved.

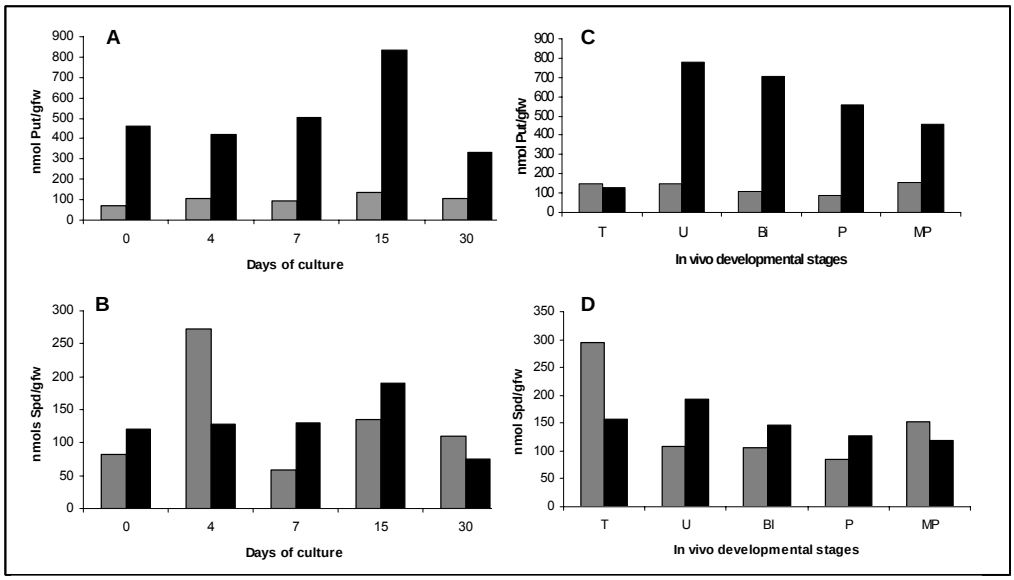

Figure 2- Free (grey) and bound (black) polyamine content (nmol PA/g fw) during the different stages of *in vitro* maize microspore androgenesis (A and B) and *in vivo* maize pollen development (C and D). A and C= Put, B and D= Spd.

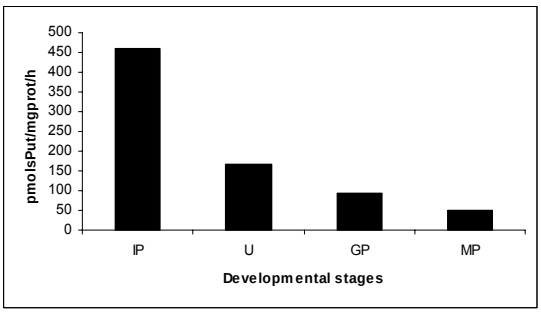

Figure 3- TGase activity (pmol Put/mg prot.h) of maize pollen in different developmental stages.

IP= cold-induced microspores;

U= uninucleate microspores;

GP= germinated pollen; MP= mature pollen.

3. Transglutaminase immunodetection

3.1 Western blot analysis

Using an antibody against chloroplast TGase [12], the same maize pollen development stages as in Figure 2 were analyzed by western blot. In all cases except with germinating pollen, a strong band of about 160-180 kDa, was obtained. The 58-50 kDa bands that appeared in the grana chloroplasts (see previous chapter) was present in uninucleate immature pollen, indicating that the active enzyme may be present in this stage of active division. Interestingly, these 58-50 kDa bands were absent in the immature pollen before cold treatment and reappeared in 3-day cultured *in vitro* pollen, when androgenesis begins (see Figure 1). In mature pollen, only the 160-180 kDa band appeared. Finally, with mature pollen induced to germinate, a very faint band of ~150 kDa was present, (Figure 4). As previously commented, the constant presence of a multimeric form indicates that the enzyme is always present and accumulated in the cell, but is predominantly active in immature maize pollen *in vivo* and *in vitro*.

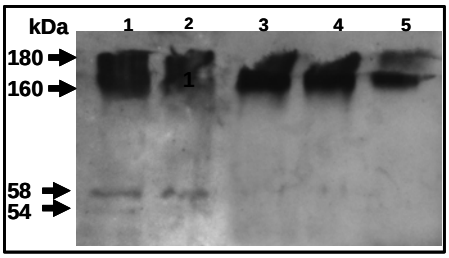

Figure 4- Western blot analysis of protein extracts from maize pollen microspores. 1, *in vivo* uninucleated microspore; 2, 10-days *in vitro* cultured microspore; 3 cold-induced microspore; 4, mature pollen; 5, germinated pollen

4.2 Cellular localization of TGase in maize microspores

For the cellular localization of the enzyme in immunocytochemical experiments, the anti-TGase antibody used for immunoblot experiments was applied to both thin and ultrathin sections of cold pre-treated microspores, non-induced microspores and mature pollen grains embedded in Unicryl (BBInt.). Bound antibody was detected in thin sections (1μm) after the application of a secondary anti-chicken antibody conjugated to Cy3, using a Zeiss Axioplan epifluorescence microscope under green excitation. Immunolocalization at the ultrastructural level was carried out on ultrathin sections (70nm) of the same samples after incubation with the primary antibody and with a 20nm gold-conjugated secondary antibody. Observations were performed in a JEM1011 TEM at 80kV.

Morphological and ultrastructural features of maize microspores during androgenesis induction have been previously described in a highly androgenic maize line [13]. The numerous microspores present in the anther before and after induction were irregularly-shaped and in close contact after LM observation (Figs 5a and 6a). Most microspores had the morphological characteristics of the late uninucleate microspore stage, with a large cytoplasmic vacuole, and a single nucleus often localized at the periphery of the microspore. The cytoplasm frequently formed strands at the edges of the microspore, close to the microspore wall.

Mature pollen grains were larger in size than microspores (Fig. 7a). The cytoplasm was denser than those of the uninucleate pollen grains, and normally filled the whole pollen grain. Numerous starch granules were present in the cytoplasm of the vegetative cell.

Auto fluorescence of the cytoplasm and the pollen walls was also seen by FM under ultraviolet irradiation (Figs. 5b, 6b and 7b). Immunofluorescence staining of TGase (Figs. 5c, 6c and 7c) in serial sections observed by FM under green irradiation is seen as brilliant red spots in the cytoplasm of all

samples. There was only slight background signal in negative controls without the primary antibody in serial sections (Figs. 5d, 6d and 7d). The intensity of the immunolocalization signal was higher in microspores (particularly in cold-induced microspores) than in mature pollen grains, where the level of labelling was relatively low. These results are in agreement with those reported above regarding TGase activity determined by *in vitro* enzyme assays at the same stages.

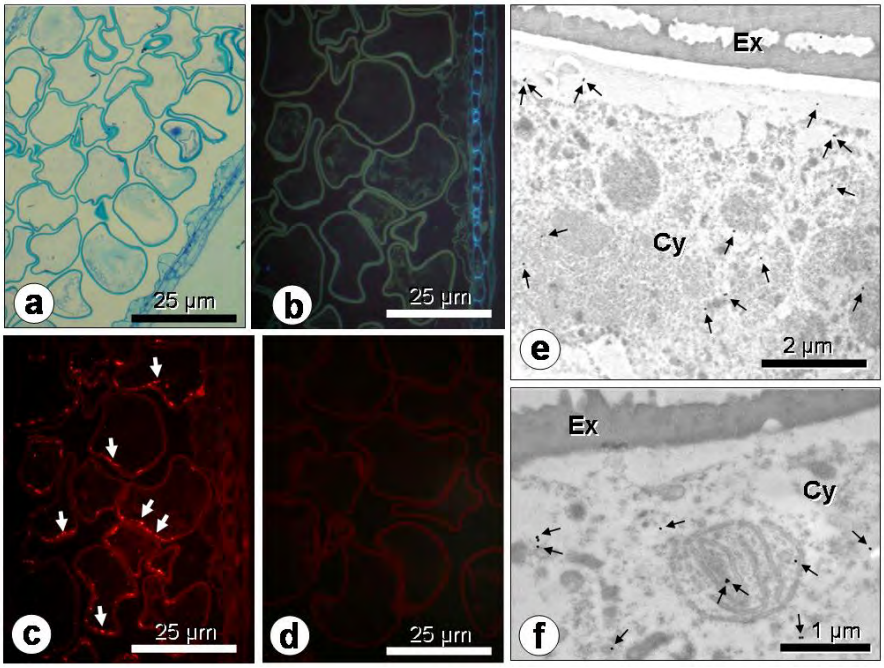

Figure 5

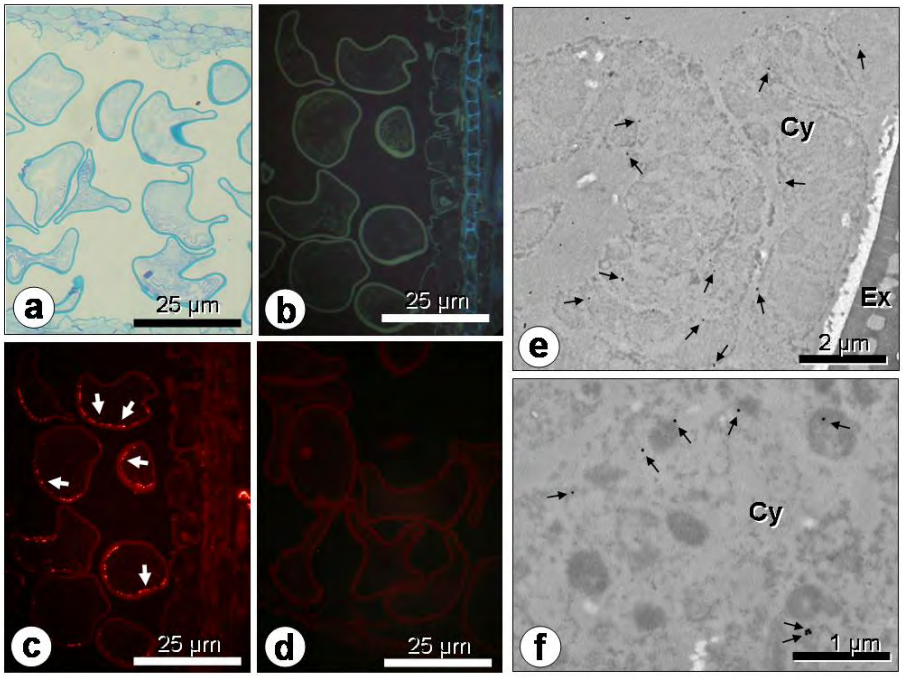

Figure 6

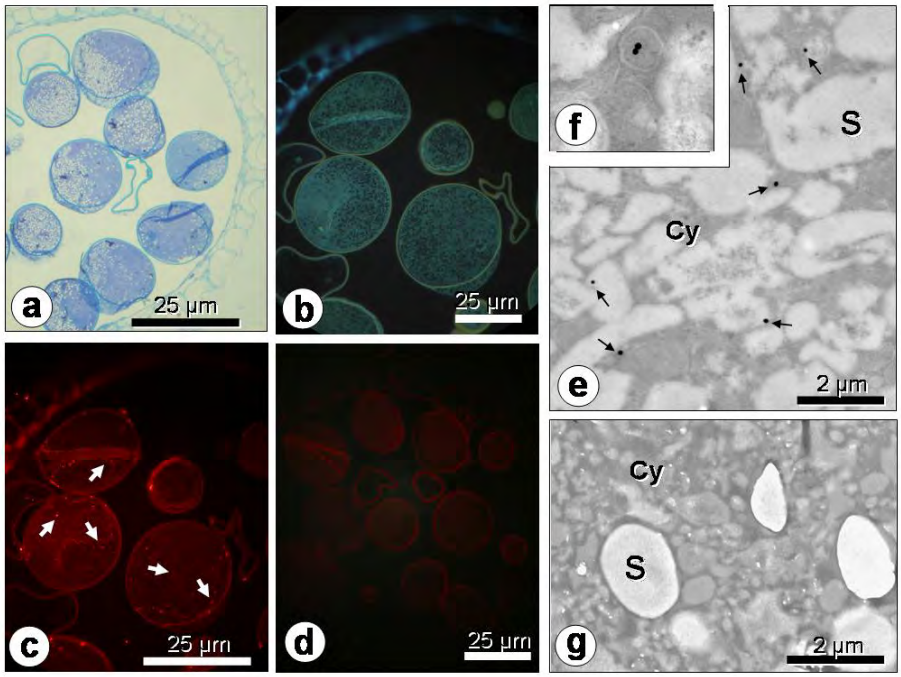

Figure 7

Figures 5, 6 and 7- Maize microspores before cold induction (Fig 5), after cold induction (Fig 6), and mature maize pollen (Fig 7). **a-b:** general view of semithin sections after Toluidine blue staining (**a**) and observation of autofluorescence by uv irradiation (**b**). **c:** semithin section after fluorescence immunolocalization with anti-TGase antibody (signal pointed by arrows) under green excitation. **d:** negative control as **c**, but omitting the primary antibody. **e-f:** TEM immunolocalization of TGase on ultrathin sections. **g:** negative control. Gold particles are marked by arrows. Cy: cytoplasm, Ex: exine, S: starch

At the ultrastructural level, in the cytoplasm of both cold-induced and non-induced microspores there were few organelles and a low density of ribosomes. The pollen wall was composed of a thin intine and a well structured exine. In both types of microspores, labelling was mostly in poorly-differentiated organelles and in membranes or associations of membranes in the cytoplasm strands close to the pollen walls (Figs. 5e, f, and 6e, f). In the mature pollen grain, the cytoplasm had a low intensity of labelling, mainly localized in young organelles or in association with membranes. The numerous starch granules present in the cytoplasm of the vegetative cells were devoid of gold particles (Figs. 7e, f), as also were the cell walls and the negative control sections without the primary antibody (Fig. 7g).

TGase has been described as a membrane-associated protein in leaves, isolated chloroplasts and thylakoids from maize and Helianthus [12], [14]. In Malus domestica pollen [9], the addition of detergents to the extracts produces an increase in TGase activity, supporting the putative association of the enzyme and/or the substrates to membranes. The results presented here clearly localize the enzyme in association with membranes and undifferentiated organelles. However, the low concentration of the enzyme, as cited in certain plant tissues [9], and the difficulty in preparation of maize microspore samples for electron microscopy means that further identification of TGase is needed, with specialised preparation techniques (i.e. cryotechniques) in order to preserve protein reactivity to antibodies and to prevent extraction of cytoplasmic components during dehydration and embedding.

Future investigation includes the co-localization of TGase and polyamines in sections in order to confirm the results indicated here. A parallel study of cytoskeleton components in maize microspores/pollen and other potential substrates for the enzyme, as previously documented in Malus [9] would help to increase our knowledge of the biological role of TGases and polyamines in androgenesis induction and pollen morphogenetic processes

Acknowledgements. These studies are supported by the Spanish MEC projects BFI2003-003318 and BFU2006-15115-CO2-01 (in Barcelona) and AGL2003-00719 and BFU2004-00601/BFI (in Granada).The authors gratefully acknowledge the technical support of C. Martinez from Granada.

References

[1] D. Barloy, L. Denis and M.Beckert, Maydica **34**, 303-308 (1998)
[2] J.C.Mitchell and J.F.Petollino, J. Pl. Physiol. **137,** 530-536 (1991).
[3] C. Nistch, S. Andersen, M.Godard, M.G. Neuffer and W.F. Sheridan, In: E.D.Early and Y.Demarly (eds) Variability in plants regenerated from tissue culture .New York, Preager (1982) pp. 69-91.
[4] J.C..Mitchell and J.F. Petollino, J.Pl. Physiol. **137,** 530-536 (1991).
[5] A.W.Galston and R.Kaur-Sahwney, pl. Physiol. **94,** 406-410(1990).
[6] A.F. Tiburcio, X.Figueras, I.Claparols, M.Santos and J.M.Torné Pl. Cell Tissue Org. Cult. 27-32 (1991).
[7] J.M.Torné, I.Claparols, X.Figueras and M.Santos, Pl.Cell Physiol. **34 (2),** 371-374 (1993).
[8] N.Boget, J.M.Torné, L.Willadino and M.Santos, Pl.Cell Tissue Org. Cult. **40,** 139-144 (1995).
[9] S. Del Duca, A.M. Bregoli, C.Begamini and D.Setrafini-Fracassini, Pl. Physiol. Biochem. **37,** 1-11 (1997).
[10] M.Santos, N.Boget and J.M.Torné, Pl.Growth Reg. **16,** 19-26 (1995).
[11] L.Willadino, T.R.Camara, m.Santos and J.M.Torné, Pesq. Agropec. Bras. **11,** 1313-1318 (1995).
[12] E. Villalobos, J.M. Torné, J. Rigau, I. Ollés, I. Chaparols and M. Santos. Protoplasma **215,** 155-163 (2001).
[13] J.D. Alché, A.J. Castro, M. Solymoss, B. Barnabas and M.I. Rodríguez-García. J. Plant Physiol**. 156(2),** 146-155 (2000).
[14] S. Del Duca, l. Dondini, M. Della Mea, P. Muñoz de Rueda and D. Serafín-Fracassini. Plant Physiol. Biochem. **38 (69**), 429-439 (2000).