Cultivos Tropicales, 2016, vol. 37, special edition, pp. 111-117

ISSN impreso: 0258-5936
ISSN digital: 1819-4087

Ministerio de Educación Superior. Cuba
Instituto Nacional de Ciencias Agrícolas
https://ediciones.inca.edu.cu

DOI: 10.13140/RG.2.1.4562.3926
http://dx.doi.org/10.13140/RG.2.1.4562.3926

CITOTOXIC ACTIVITY OF AN ASPARTIC PEPTIDASE FROM *Salpichroa origanifolia* AGAINST THE INFECTION CAUSED ON GREEN ZUCCHINI (*Cucurbita maxima,* VAR. ZAPALLITO) BY *Phytophthora capsici*

Citotoxicidad de una aspartil peptidasa de *Salpichroa origanifolia* frente a la infección causada por *Phytophthora capsici* en zapallitos verdes (*Cucurbita maxima*, var. zapallito)

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ABSTRACT. *Phytophthora capsici* is a phytopathogenic agent that causes significant losses in crops of economic interest. The chemicals traditionally used to fight these pathogens cause adverse effects on health and the environment. Native plants represent an alternative source of natural antifungal metabolites. In our laboratory we have studied a perennial herb, *Salpichroa origanifolia*, native to the northern and central Argentina whose ovoid fruits are edible berry. An aspartyl peptidase from the crude extract of the ripe fruit of this species was purified by ion exchange chromatography using a batch process. The enzyme was called salpichroín. In this work the antifungal effect of the aspartyl peptidase on *P. capsici* was studied. It was obtained that salpichroín had a high cytotoxic effect *in vitro* on strains of *P. capsici* (MIC: 1.2 µmol L^{-1}). To evaluate the enzyme effect on the development of *P. capsici in vivo*, inoculation controlled bioassays on green zucchini (*Cucurbita maximum* var Zapallito) were performed. The fruit inoculated with the phytopathogen and salpichroín remained asymptomatic for seven days.

Key words: fungicides, pathogens, peptidase

RESUMEN. *Phytophthora capsici* es un oomicete patógeno que causa importantes pérdidas en la producción de cultivos de interés agroeconómico. Los agroquímicos utilizados tradicionalmente para combatir estos fitopatógenos causan efectos adversos sobre la salud y el medio ambiente. Las plantas de la flora autóctona representan una fuente alternativa de metabolitos antifúngicos naturales. En nuestro laboratorio hemos estudiado la especie *Salpichroa origanifolia*, una hierba perenne autóctona del Norte y Centro de Argentina cuyos frutos, en forma de baya ovoide, son comestibles. A partir del extracto crudo de los frutos maduros de esta especie se purificó por cromatografía de intercambio iónico mediante un proceso en batch, una aspartil peptidasa a la cual denominamos salpichroína. El objetivo de este trabajo fue estudiar el efecto antifúngico de la enzima sobre el patógeno *P. capsici.* Salpichroína presentó un elevado efecto citotóxico sobre *P. capsici* en los ensayos *in vitro,* con un valor de CIM de 1,2 µmol L^{-1}. Para evaluar el efecto de la enzima sobre el desarrollo de *P. capsici* en vegetales, se realizaron bioensayos de inoculación controlada de zapallitos verdes (*Cucurbita máxima* var Zapallito), observándose que los zapallitos inoculados con salpichroína junto con el fitopatógeno permanecieron asintomáticos durante siete días.

Palabras clave: fungicidas, organismos patógenos, peptidasas

INTRODUCTION

Phytopathogenic fungi are the main cause of infectious diseases in plants. The control of these fungi with synthetic fungicides is still the most important plant breeding measure to increase crop yields. However, the massive and sometimes

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indiscriminate use of these products has increased the resistance of phytopathogenic organisms as well as causing adverse effects on human health and the environment (1). Within this framework, the search for safer antimicrobial agents that can replace or reduce the current use of agrochemicals is essential. The development of sustainable agriculture has led researchers from all over the world to seek new compounds for the control of diseases whose activity and environmental safety is adequate (2). In this sense, natural alternatives are being evaluated, among which is the use of plant extracts. With these extracts, promising results have been obtained since the antimicrobial activity of different plant extracts has been demonstrated *in vitro* and *in vivo* (3, 4, 5).

Plants have developed a sophisticated innate immune system in response to invading pathogens. Thus, plant defense involves a variety of cellular mechanisms, among which we can mention the rapid generation of reactive oxygen species (ROS), the induction of the hypersensitive response (HR) and the production of small molecules (phytoalexins) and proteins with antimicrobial activity. Induction of genes encoding endopeptidases with different catalytic mechanisms has also been described (6, 7). Cysteine peptidases are involved in many aspects of plant development and physiology including senescence, embryogenesis, flower development and response to different types of environmental stress (8). In addition, the pharmacological action of plant cysteine peptidases has been recognized in investigations as potential drugs to combat bacterial and fungal diseases (9, 10). On the other hand, aspartic peptidases (APs) of vegetal origin have been described as a fundamental part of the defense mechanism of plants against infection by pathogenic microorganisms (11).

In our laboratory we have studied the species *Salpichroa origanifolia*, a perennial grass indigenous to the North and Center of Argentina, Uruguay and Brazil. The fruits of this species are shaped like ovoid berry, and they are edible and commonly called rooster eggs. In crude extracts of mature fruits a peptidase was found. From said extract it was purified by anion exchange chromatography and characterized this AP which we call salpichroin (12).

On the other hand, the oomycete *P. capsici* has been described as causing late rot and blight of a wide variety of hosts, including many members of the family *Solanaceae* and *Cucurbitaceae* as well as *Fabaceae*. among the crops of agroeconomic interest affected are potato, sweet potato, tomato, pepper, eggplant and zucchini crops. Therefore, the present work aimed to determine the cytotoxic activity of AP purified from mature fruits of *S. origanifolia* against infection caused by *P. capsici* in Green zucchini (Cucurbita maxima, var. Zapallito).

MATERIALS AND METHODS

Plant material: Mature fruits of the *Salpichroa origanifolia* (Lam.) Baill species were used as a source of proteolytic enzymes, collected in Luján town, Buenos Aires province, Republic of Argentina. The ripe fruit is an ovoid, soft, whitish berry with sweet taste.

Preparation of crude extract: The fruits were triturated with ethanol at -8 °C. The precipitate was resuspended in 50 mM potassium phosphate buffer of pH 7,0 (4 °C) and a 15 % (w/v) extract was obtained. This enzyme preparation was called crude extract (CE).

Determination of proteolytic activity: The determination of proteolytic activity was performed using casein as substrate (13). The reaction was started by adding 100 µL of the enzyme solution to 900 µL of 0,5 % (w/v) casein solution in 50 mM potassium phosphate buffer of pH 6.0 and then 15 minutes incubation at 40 °C, the reaction was stopped with the addition of 1 mL of 5 % (w/v) TCA. The suspension was allowed to stand for 20 minutes in an ice bath and then centrifuged for 10 min at 14000 rpm. The absorbance of the supernatant was measured at 280 nm to determine the soluble peptides obtained by proteolytic digestion. The Caseinolytic Activity Unit (Ucas) was defined as the amount of enzyme required to produce an increase of one unit of absorbance per minute at 280 nm under the conditions of the assay

Determination of Protein Concentration: The protein concentration was estimated by the Bradford method (14) using bovine serum albumin as the standard for the construction of the calibration curve.

Purification of AP: The purification was carried out by anion exchange chromatography with a DEAE-Sepharose Fast Flow (Sigma) resin equilibrated in 50 mM potassium phosphate buffer of pH 7.0. Proteins were eluted with a linear gradient of NaCl (0,0-0,6 M) with a constant flow of 1 mL min^{-1}. The active fraction was desalted by molecular exclusion chromatography with Sephadex G-10 resins and stored at -20 °C for further testing. The active fraction eluted from the chromatographic column was analyzed by SDS-PAGE on polyacrylamide gels (15 %),

under reducing and non-reducing conditions and at room temperature (15). The gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250) and silver (16). Low molecular weight standards (Low Molecular Weight Range Sigma Marker product No. 3913M) were employed in the SDS-PAGE electrophoresis. For the zymograms Kleiner and Stetler-Stevenson technique was applied (17). After the electrophoretic run, the gel was incubated overnight in a sodium citrate buffer (pH 4,0) with 20 mM CaCl 2 at 40 °C. After washing, the gel was stained with CBB R-250 for zymography analysis. The development of a clear band on the blue gel background indicated the presence of peptidase activity.

***In vitro* antifungal activity**: The phytopathogen used in this study (*P. capsici*) was isolated and characterized by the Phytopathology research group of the National University of Luján, from locally produced vegetables, from the Northeast area of Buenos Aires province. To determine the antifungal activity of salpichroin, 0,5 cm diameter agar disks with mycelial growth of the fungal strain studied were incubated with salpichroin (2,4 µmol L^{-1}), 50 mM sodium phosphate buffer of pH 7, or water for 24 hours. The agar plates were then placed in Petri dishes containing V8 medium (34 % juice of eight V8® vegetables and 3% agar) specific for the growth of *P. capsici*. One agar plate per plate was placed and the assays performed in duplicate. The plates were incubated at room temperature for ten days. After three and ten days the inhibitory effect of the enzyme on mycelial growth of the fungal species was monitored.

Minimum inhibitory concentration (MIC) was defined as the minimum concentration of salpichroin that produced complete inhibition in the growth of P. capsici. For this purpose, a 0.5 cm diameter agar disc was incubated with mycelial growth of the fungal strain with different concentrations of the enzyme (0,0-0,6-1,2- 2,4 µmol L^{-1}) In V8 liquid medium (10 %) at 25 °C for 48 hours. The MIC was determined visually and corresponded to the lowest concentration of salpichroin in which there was no mycelial development of the oomycete.

Controlled inoculation of P. capsici phytopathogen on Green zucchini: Controlled inoculation bioassays were performed to evaluate the effect of salpichroin on the development of *P.capsici* in vegetables. Green zucchini (Curcubita maxim var zapallito) were used, purchased from stores in the city of Luján, Buenos Aires. First the vegetable surface was carefully washed with water, so as not to cause any injuries. 3 mm deep wounds were made in the equatorial region of the plant with a sterile dissection needle to promote infection.

The bioassay consisted in placing an agar disc of the V8 culture medium with mycelium of *P. capsici* in active growth on the wound made in the skin of the clean zapallitos. 50 µL of a sterile solution of salpichroin (2,4 µmol L^{-1}) or the same volume of sterile water was placed on the culture for the positive control on the culture. For the negative controls, sterile agar discs were used, without either the enzyme or the pathogen. For each treatment three replicates were made and the complete assay was repeated twice. All the vegetables were incubated at 25 °C in a moist chamber and with alternating light.

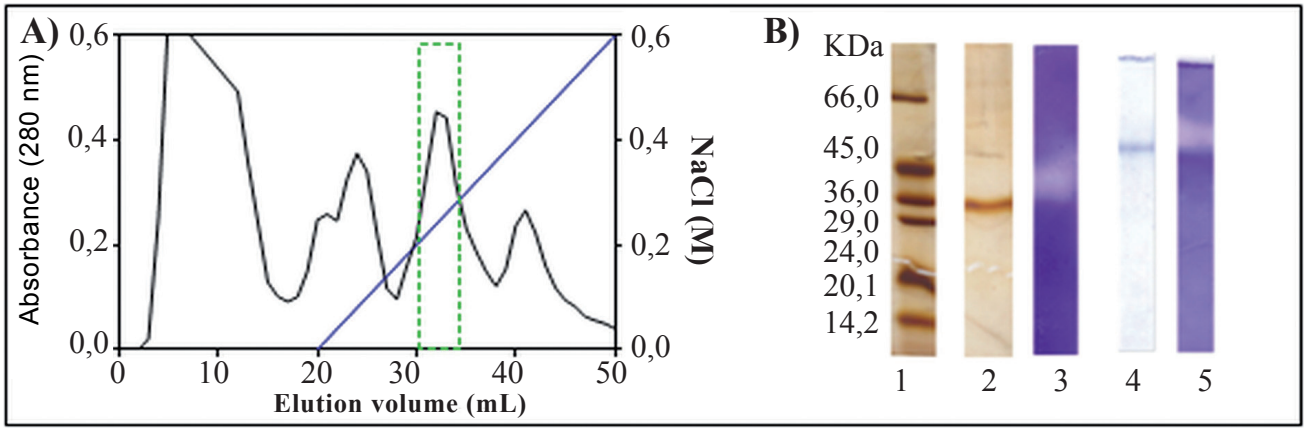
Infection development was evaluated daily from two days after inoculation and for seven days, recording as the percentage of infected wounds per replicate and calculating the mean and standard deviation for each treatment.

RESULTAS AND DISCUSSION

Isolation and characterization of pure enzyme

An CE of mature fruits of Salpichroa origanifolia with a protein concentration of 0.5 mg mL-1 and a proteolytic activity of 145 Ucas mL^{-1}. Although in previous work the enzyme was purified by an FPLC system (12), in this work the purification from the CE was performed in batch using anion exchange chromatography with a DEAE-Sepharose resin. This technique allowed to obtain a greater pure enzyme biomass necessary to be used in the tests of microbial inhibition. In the chromatographic profile five absorbance peaks were observed at 280 nm of which only one showed caseinolytic activity (Figure 1A). The active fraction obtained was analyzed by SDS-PAGE electrophoresis under reducing and non-reducing conditions. A single band with apparent molecular weight of 32 kDa when the sample was prepared under non-reducing conditions (Figure 1B, lane 2), no differences in electrophoretic profile were observed when the sample was prepared under reducing conditions and heated to 90 °C (data not shown). The purity of the enzyme was also evaluated by native electrophoresis and the presence of a single protein band was observed (Figure 1B, lane 4). In the same way, in the corresponding zymogram a single band with relative mobility similar to that obtained in the gel was observed, indicating the homogeneity of the purified enzyme.

According to the results obtained in the electrophoretic assays, the molecular weight of the enzyme salpichroin in 32 kDa was estimated, which is of the same order as that obtained for other APs of plant origin such as tomato APs (37 kDa) (18) and tobacco (36-40 kDa) (19), potato AP (38 kDa) (20) and *Ficus racemosa* AP (44 kDa) (21).



B: lane 1: low molecular weight marker (M3913, Sigma), lane 2: active fraction of anion exchange chromatography, tinted with silver, lane 3: zymogram of said fraction, lane 4: native electrophoresis (10%) fraction Purified, lane 5: native electrophoresis (10%) zymogram purified fraction

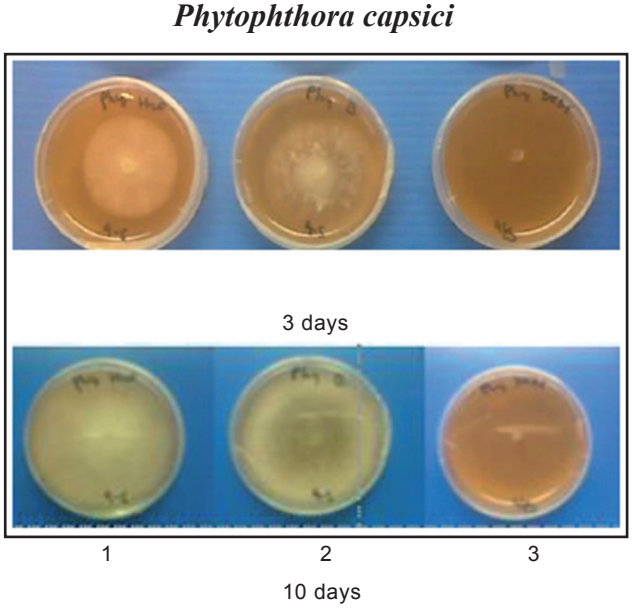
Figure 1. Anion exchange chromatography (DEAE-Sepharose Fast Flow) of crude extract of *Salpichroa origanifolia* (A) and SDS-PAGE electrophoresis (15 %) under non-reducing

Antifungal activity of salpichroin

The presence of multiple biological activities associated with peptidases, including antifungal activity, has been reported in the literature. It was found that bromelain purified from pineapple stems was a potent inhibitor of plant fungal pathogens and that this activity was associated with its proteolytic activity (7). On the other hand, it was reported that the antifungal activity of potato APs was related to the presence of PSI in the mature enzyme (22). For this reason the study of the antifungal activity of salpichroína was initiated.

First, the inhibitory effect of the enzyme on the mycelial development of *P. capsici* (Materials and Methods section) was evaluated. In the microbiological assays, the enzyme was found to have a strong cytotoxic effect on *P. capsici* strains as no development of the mycelium was observed after incubation in the enzymatic solution during the ten days of the assay (Figure 2). As expected, mycelial development of the strains was observed on the plates containing the disks submerged in water and in buffer.

These results coincide with those reported by other authors (22) for potato leaf and potato APs, StAP1 and StAP3, which showed fungicidal action against Phytophthora infestans and Fusarium solani, pests that infect potato crops. In addition, they found that the antifungal activity of StAp1 and StAP3 was related to the presence of PSI, a specific domain of APs, which exhibits high structural homology with saposin-like proteins (SAPLIPs) and acts destabilizing the oomicete phospholipid membrane, causing pores formation and loss of cell viability of the pathogen (22).



Treatment 1 corresponds to the preincubation of a disk with fungal mycelium in water, the 2 in 50 mM solution of potassium phosphate buffer of pH 7.0 and the 3 in salpichroin solution

Figure 2. Development of P. capsici in V8 culture medium incubated for 3 and 10 days at room temperature

Thus, the fungicidal activity of salpichroin could be due to the potential presence of the PSI domain in its active form.

Once the cytotoxic activity of the enzyme on *P. capsicium* was checked, the MIC of salpichroin was determined on the *in vitro* growth of the microorganism (Figure 3). The MIC value was 1. µmol L^{-1}, which is of the same order as the MIC values reported for StAP1 and StAP3 on *P. infestans* (22).

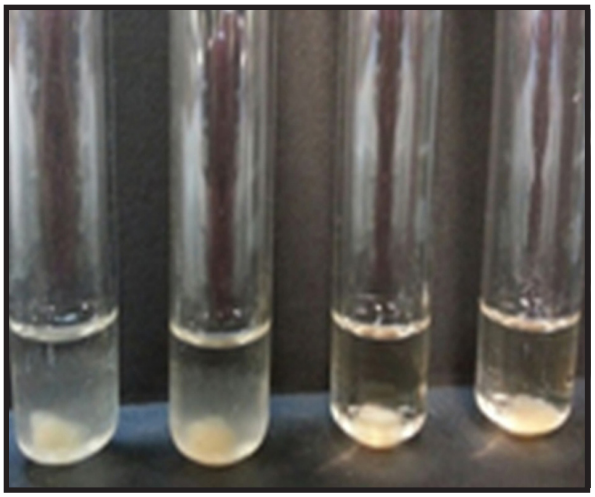


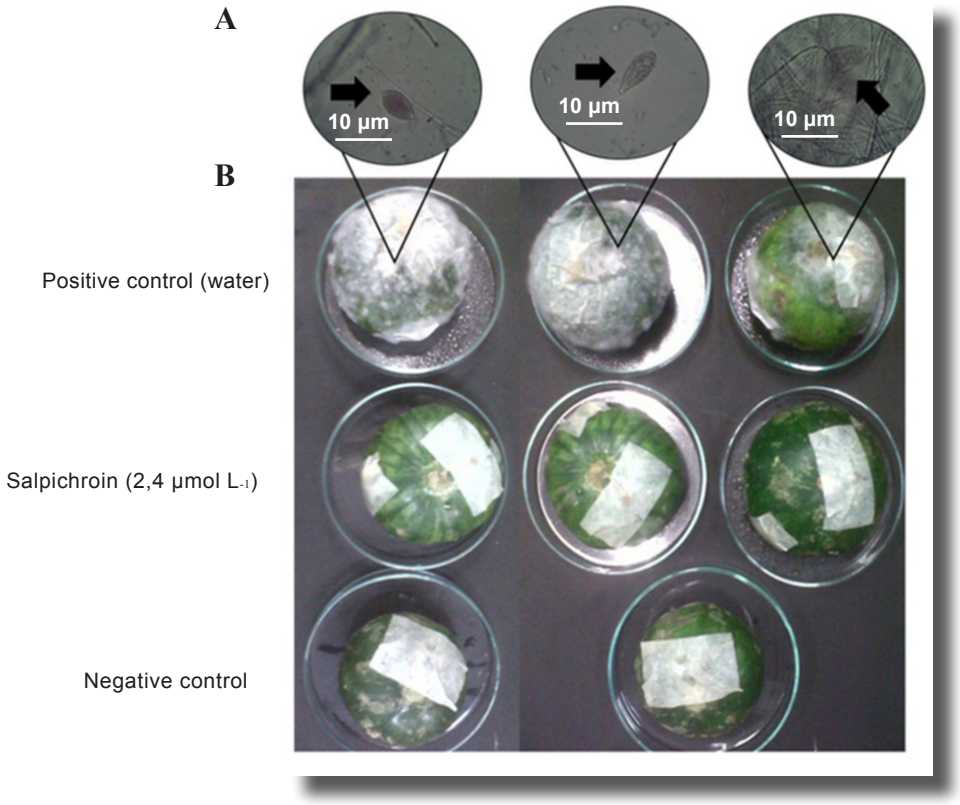
Figure 3. Minimum inhibitory concentration of salpichroin (1,2 µmol L^{-1}) on *P. capsici*

Based on these results we can infer that salpichroin presents interesting antimicrobial properties on the *in vitro* growth of the *P. capsici*, oomycete phytopathogen that generates great losses in the production of vegetables like zucchini, peppers and eggplants among others.

In order to test the *in vivo* cytotoxic activity of the enzyme, controlled inoculation experiments of salpichroin on green zucchinis infected by *P. capsici* were carried out.

The bioassay consisted in placing a disk of agar with mycelium of *P. capsici* in active growth in the skin of clean zucchini. A sterile solution of salpichroin (2,4 µmol L^{-1}) or the same volume of sterile water was placed on the culture for the positive control on the culture. For the negative controls only one agar disc was used without any pretreatment. It was incubated at 25 °C in a humid chamber for seven days and the severity of the lesion was recorded. After incubation, pathogenicity signs were observed on all positive controls.

Samples taken from the surface of these fruits confirmed that it was *P. capsici* due to the presence of sporangia with typically long pedicels (Figure 4A).



A: image at optic microscopy (40x) de *P. capsici* taken of the surface in the positive controls

B: bioassay constituted by positive control (water and *P. capsici*), samples (Salpichroin and *P. capsici*) and negative control (water and sterile agar, without P. capsici) on green zucchini

Figure 4. Image of one of the replicates of three zucchins used in the biocontrol experiment

The zucchini on which the enzyme solution was placed showed no signs of infection and just as the negative controls remained asymptomatic (Figure 4B). This behavior was repeated in all replicates of the trial.

According to these results, salpichroin peptidase aspartyl isolated from mature fruits of *S. origanifolia*, could control the infection of *P. capsici* on green zucchinis, in the postharvest stage.

CONCLUSIONS

- Because the control of phytopathogenic fungi with synthetic fungicides has increased the resistance of these organisms, natural alternatives such as plant extracts are being evaluated. Promising results have been obtained since the antimicrobial activity of different plant extracts has been demonstrated *in vitro* and *in vivo*. In particular, peptidases of plant origin have been shown to possess antimicrobial activity.
- In this work we have purified an AP of mature fruits of *S. origanifolia* by ion exchange chromatography using a batch process. In addition, it has demonstrated that the purified enzyme (salpichroin) has a cytotoxic effect on the phytopathogen *Phytophthora capsici* as it inhibits its growth. This could be demonstrated both *in vitro* and *in vivo*, by performing controlled inoculation trials on green zucchini.
- From these results we can affirm that salpichroin can control the infection of *P. capsici* on green zucchinis probably due to the antifungal activity of the PSI of APs that can interact with the cell membrane of the oomycete causing the loss of cellular viability.
- These results are very promising since the search for natural compounds of plant origin for the control of pests that affect crops of agrifood importance is an issue of social and economic interest.

ACKNOWLEDGMENTS

The results of this work are part of the doctoral thesis of Ing. Gabriela Rocha. We are grateful for the funding received from the Department of Basic Sciences of the National University of Luján, the Research Grants Program of the Scientific Research Commission of Buenos Aires Province (CIC 2013) and the International Networks Program of the Secretariat for University Policies of the Ministry of Education of the Argentine Republic.

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Received: May 15th, 2015
Accepted: September 15th, 2016

SPECIAL NUMBER

This issue of the magazine is dedicated to the X International Congress of Plant Biotechnology (BioVeg2015)

Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial

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