Emir. J. Food Agric. 2011. 23 (6): 533-541
http://ejfa.info/

Proteolytic response of *Zea mays* L. hybrids to citric acid treatment

Mauro Manuel Martínez Pacheco[1*], Rosa E. Martinez Muñoz[1], Oscar A. Ron Echeverria[1], E. Venegas-Gonzalez[2] and Mario A. Cepeda-Villegas[2]

[1]Instituto de Investigaciones Quimico Biologicas. Universidad Michoacana de San Nicolas de Hidalgo. Ed. B-1; Cd. Universitaria. Francisco J. Mujica s/n. Col. Felicitas del Rio. C.P. 58060 Morelia, Mich. México. [2]Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias, Campus Uruapan, Mich. México

Abstract: Hybrids of *Zea mays* L. (Buffalo, Falcon, H360 y HV313) were treated with citric acid (2000 ppm). The grain yield, the soluble protein and the proteolytic activity were monitored in the physiological maturity stage of the crop. The citric acid was applied before the appearance of the flag leaf, induced an increase in the production of the grain from 540 to 945 kg·ha^{-1}, in the soluble protein from 1.39 to 4.2 mg·mg d·w^{-1} and into the proteolytic activity from 2 to 12 times in the Falcon, H360 and HV313 hybrids, while the Buffalo hybrid responded with less intensity to the treatment with citric acid. In the H360 hybrid treated with citric acid, an increase in the proteolytic activity of aspartyl serine proteases was observed. The results indicate that citric acid induces differentially, the proteolytic activity and the vigor of the corn hybrids analyzed.

Key words: corn, citric acid, proteolytic activity.

دراسة بروتين (Proteolytic) للذرة الشامية المهجنة لمعاملات حمض الستريك

ماورو مانويل مارتينيز باتشيكو [١] ، روزا مارتينيز مونيوز هاء [١] ، أوسكار ألف رون اتشيفيريا [١]، إ. فينيغاس ـ غونزاليس [2] وماريو ألف سيبيدا فيليجاس [2]

[1]معهد بحوث الكيمياء البيولوجية. يونيفرسيداد دي سان نيكولا Michoacana دي هيدالغو. أد B ـ ١ ، سيوداد Universitaria. فرانسيسكو جيه. موخيكا ق / ن. فليسيتاس العقيد ديل ريو C.P. ٥٨٠٦٠ موريليا ، ميتشواكان. المكسيك; [٢] المعهد الوطني للزراعة الغابات ، الثروة الحيوانية والبحوث الزراعية، حرم Uruapan، ميتشواكان. المكسيك

الملخص: تم معاملة نبات الذرة الشامية المهجنة (Fuffalo, Falcon, H360 y HV313) بحمض الستريك ٢٠٠٠ جزء بالمليون ، إنتاج محصول الحبوب ومسيلة البروتين ونشاط (Proteolytic) تم رصدها في مراحل نضوجها الفسيولوجي للمحصول . تم إضافة حمض الستريك قبل ظهور الأوراق الأولية مما أدى إلى زيادة في إنتاج الحبوب من ٥٤٠ إلى ٩٤٥ كيلوجرام للهكتار ومعدل سيولة البروتين من ١,٠٣٩ إلى ٤,٢ ملغرام لكل وزن جاف . ومعدل نشاط (Proteolytic) من ٢ إلي ١٢ ضعف من صنف الفالكون والصنف المهجن (H360) والصنف (HV313) أما من صنف الهجين بافلو كان معدل الاستجابة اقل لحمض الستريك وفي الصنف المهجن (H360) المعالج بحمض الستريك لوحظ زيادة في نشاط (Proteolytic) وتشير النتائج أن حمض الستريك يحفز بشكل مختلف نشاط (Proteolytic) والمساهمة في حيوية الذرة الشامية المهجنة.

*Corresponding Author, *Email*: mpacheco@umich.mx
Received 15 March 2011; Revised 08 May 2011; Accepted 08 May 2011

Introduction

The growing demand for agricultural products produced without pesticides, has motivated the development of diverse strategies for controlling disease and the pathogens to increase the crops yield (Lyons et al., 1995; Kuc´, 1999). The pathogen attack may be present from the first stages of development of crop and heaviest damage is strongly related with the stress level of the plant, both in the flowering as in the filling out of the corn grain stages. The inductors of plant acquired systemic resistance are an alternative. This is induced with the application of metabolites involved in some stage of development of the plant defense response, for example, the exogenous application of methyl jasmonate in barley crops, or benzothiadiasole and 2,6-dichloroisonicotinic acids on wheat crops (Mitchell and Walters, 1995; Gorlach et al., 1996). β-ionona, 3-isobutyloyl-β-ionona and 3-n-butyloyl-β-ionona in tobacco induced resistance to the blue mold pathogen, *Peronospora tabacina* Adam (Salt et al., 1986; Kuc´ and Tuzun, 1990). Molecules derived from pathogen such as glycans have been applied successfully to diverse vegetable crops as well. Doubrava et al. (1988) demonstrated that oxalic acid a carboxy acid, induced systemic resistance in cucumbers and in leaves of the kiwi fruit against *Colletotrichum lagenarium* and *Sclerotinia sclerotiorum*, respectively (Reglinski et al., 1997; Toal and Jones, 1999). Likewise other effects induced by these molecules have been observed in plants, such as, the application of citric acid to the rhizosphere of wild tabacco plants or its presence by radical hyper exudation of citrates in tobacco genetically modified for the over production of citric acid, induced an increase in dry weight (López Bucio et al., 2000). The function of the carboxilic acids such as citric acid in vegetable response to environmental stress is complex and beginning to be understood. Citrate is considered to be the most powerful organic anion, followed by oxalate and malate, to mobilize phosphorous in the soil (Kraffczyk et al., 1984; Bolan et al., 1994). The beneficial effect of this physical chemical action in the roots of wheat, buckwheat, legumes and triticale, can be interpreted by the formation of stable molecular complexes between carboxylic acids and metallic cations favoring the availability and absorption with increase in the vigor of the plant (Ryan et al., 1995; Pellet et al., 1996; Ma et al., 1997; Yang et al., 2000). With the exception of the analysis of soluble protein and proteolytic activity, the presence of carboxylic acid in the rhizosphere and it is effect on the vigor of the plant has been well studied. Nevertheless, it is not known if citrate have other different effect on the plant, apart from those already described, that is way the aim of this work to know if the grain yield, protein soluble and proteolytic activity on several commercial corn hybrids would inducing by citric acid when is applying to leaves. We can report that citric acid led to accumulation of soluble protein in the leaf and a differential induction of proteolytic activity in four corn hybrids used in this study.

Material and Methods

Fieldwork was done in the humid, temperate central western zone of Mexico at 19º 49´ latitude and 101 ° 01´ longitude at an average altitude of 1,828 masl. It has an average annual rainfall for 600 mm with vertisoils ground that contains over 70% clay, which has a pH that varies from 7.8 to 8.8 a low organic material content, and a production system of zero farming which permits a wider range of humidity even with low levels of precipitation.

In the Spring-Summer cycle, the following genotype hybrids of commercial corn (*Zea mays*) were evaluated: Falcon, a hybrid of the early with a period of 138 to 150 days from planting to physiological maturity; average height 240 cm with 64 to 77 days till flowering, of white grain with high protein content and good industrial use quality that has moderate resistance to stem an cob rotting. Buffalo, a hybrid of the intermediate cycle with a period of 152 to 163 days from planting to physiological maturity; average height of 235 cm with 78 to 89 days till flowering that is resistant to stem and cob rotting. HV313, a varietal hybrid of the early intermediate cycle with a period for 145 to 155 days planting to

physiological maturity, obtained from crosses between tropical and brachitic varieties; with an average height of 250 cm and 69 days till flowering. H360, a trilineal hybrid generated with 75% tropical germplasm and 25% subtropical germoplasm of white grain of the intermediate cycle with a period 158 to 163 days from planting to physiological maturity.

Monohydrated citric acid with a purity of 99.8% was sprayed in doses of 2000 ppm in the stage prior to the appearance of the flag leaf. In the stage of physiological maturity, six plants from each of the four corn hybrids were collected at random for processing in the laboratory and at crop maturity (when the grain had between 14 and 16% humidity) a parcel of 8 m^2 was harvested to estimate the yield by hectare, adjusted to 12% humidity.

The corn plants were put in recipients at 4°C during the collecting. From the fragments of the vegetable organs asepsis was made with the exposition of; 15% extran, 70% ethanol, 3% hydrogen peroxide and 0.5% sodium hypo chlorite. After of asepsis process they were washed exhaustively with sterile deionized water and stored in plastic bag at a temperature for –70°C until their use.

Proteolytic activity assay

This was done with a modification of the Anson method (Anson, 1938). Briefly, 1 g of leaf was homogenized with 15 ml of 0.01 M Tris-HCl to a pH of 7.5 with 5 mM 2-mercaptoethanol and 0.5% polyvinyl polypyrrolidone ($v \cdot v^{-1}$). The crude extracts were centrifuged at 20,000g and supernatant was used as enzymatic extract for the proteolytic activity determination. The proteolytic activity was measured with denaturalized hemoglobin and it was determined that the product hydrolyzed to an absorbance of 280 nm. A coefficient of tyrosine extinction of 1250 $cm^{-1}g^{-1}$ was used to calculate the proteolytic activity, one unit being a µmol of tyrosine equivalent to min^{-1} of hemoglobin in standard condition (pH 7.5, 37°C), and is reported as $U \cdot \mu g\ prot^{-1}$.

Inhibition of proteolytic activity

Proteolytic activity several classed specifically inhibitor were used such as; 0.2 mM phenylmethylsulfonyl fluoride (PMSF: an inhibitor of serine proteases), 0.15 mM pepstatin A (an aspartyl proteases inhibitor), 10 mM ethylenediamine tetraacetic acid (EDTA: a metalloproteases inhibitor) and 2 mM *p*-chloro mercuribenzoic acid (*p*CMB: a cystein proteases inhibitor). The crude extract of the corn leaf was incubated with the inhibitors at the concentration described and the residual proteolytic activity was determined. Determination of soluble protein of the vegetable extracts was done by the Lowry method (Lowry et al., 1951).

Data analysis

Statistical package Statistica 6.0 was used to analyze the data set. The experiments were repeated twice and were done in a completely random pattern. In each experiment, six plants were used, for each variety and treatment. The evaluation of the statistical significance of the parameter was based on Student *t* test ($p = 0.5$). The information about the grain production, the proteolytic activity and the soluble protein (taken as an indicator of mass) of the corn hybrids was analyzed by Principal Component Analysis (PCA) to know their similarities and differences in their response to citric acid. To carry out the analysis, the response data of each treatment for each replica was used. The groupings obtained with the PCA are based on an unsupervised evolution of the parameters for each replica. It is assumed that where the groups are similar, the PCA will identify the top position among the groups.

Results

The Falcon, Buffalo, HV313 and H360 hybrids were evaluated to yields, proteolytic activity and soluble protein. The Buffalo hybrid presented the poor yields, being similar with or without the application of citric acid (Table 1). The Falcon, HV313 and H360 hybrids showed positive results to the application of citric acid in yield, with increase between 540 to 945 $kg \cdot ha^{-1}$. The hybrid corn that showed the most increase in yield with the

foliar application of citric acid was the Falcon hybrid, with more than 900 kg·ha^{-1}. It was also observed that citric acid induced an increase of the measured biomass for soluble protein in the HV313 and H360 hybrids (Table 1).

Citric acid induced a differential increase in the proteolytic activity in the *Z. mays* hybrids. The induction was less for the Buffalo hybrid, intermediate in HV313 and H360 while the highest induction was in Falcon (Table 1). The induction observed in proteolyic activity was found between the intervals of 2 to 12 times. The Falcon, HV313 and H360 hybrids responded better to foliate treatment with citric acid, while the Buffalo hybrid presented a modest increase (Figure 1).

Table 1. Grain yield, proteolytic activity and soluble protein of *Z. mays* treated with citric acid.

Hybrid	Citric acid (2000 ppm)	Grain yield (kg·ha^{-1})	Proteolytic activity (µU·mg prot^{-1})	Soluble protein (mg prot·mg dry weight^{-1})
BUFFALO	-	4139	6.9±0.6	4.95±0.74
	+	4222	14.3±1.7	6.34±1.74
FALCON	-	4891	6.4±0.3	4.77±0.26
	+	5836	70.0±10	9.19±1.91
H360	-	4707	8.7±2.6	4.34±0.12
	+	5349	65.7±12	6.58±1.53
HV313	-	5240	5.4±0.5	5.03±0.43
	+	5780	65.0±5	7.91±1.22

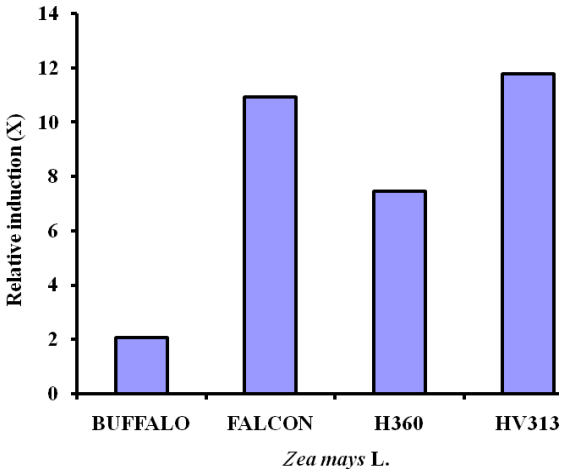

Figure 1. Relative increase of the proteolytic activity of *Z. mays* hybrids induced with citric acid.

Due to the fact that all the hybrids used gave a positive response and differential to the treatment with citric acid and to know which of them gave best response, a principal component analysis (PCA) was made. The first PC describes a total variance of 71.01% for the three variables shown in Table 1. In figure 2, three groups are shown, one, where control hybrids are found (without citric acid). The second group has a distribution that indicates that citric acid influences differentially the production of soluble protein, proteolytic activity and production of the grain, with an ample and different response in the Falcon (Ha), H360 (H) and HV313 (HV) hybrids. The third hybrid group to that citric acid no induced any effect on proteolytic activity.

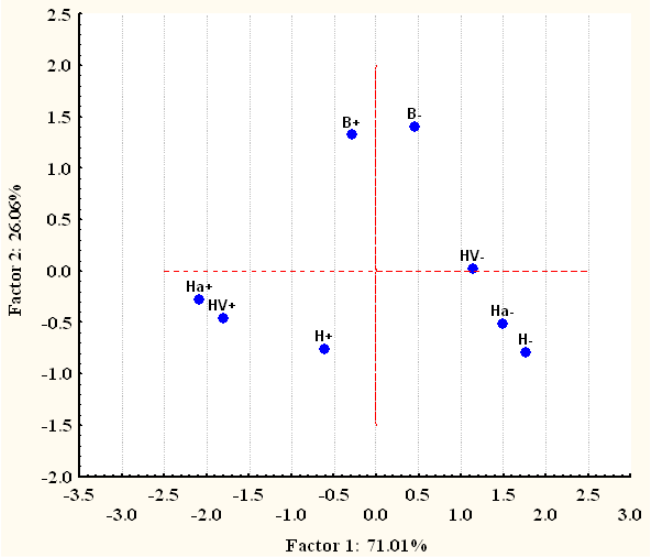

Figure 2. Principal component analysis of grain production, proteolytic activity and soluble protein in leaves of *Z. mays* hybrids treated with citric acid.
Corn hybrids, Ha (Falcon), H (H360) and B (Buffalo). (-), Control and (+), treatment with citric acid.

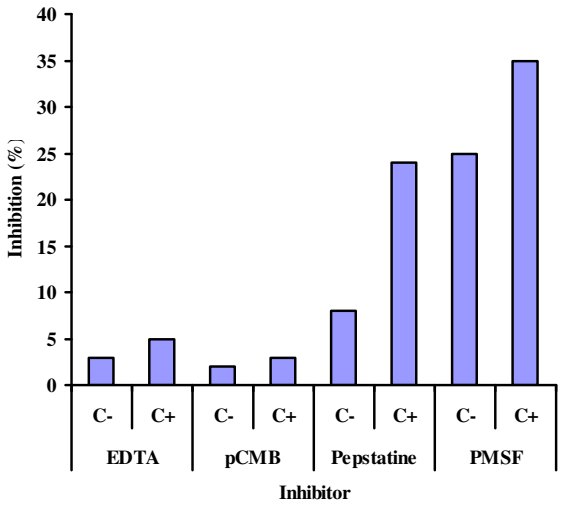

Figure 3. Effect of class specific protease inhibitor in the proteolytic activity of *Z. mays*.
Extracts from leaves of the hybrid H360 were used and the proteolytic activity was measured in the presence of PMSF (an inhibitor of serine proteases), pepstatin A (an aspartyl proteases inhibitor), EDTA (a metalloproteases inhibitor) and *p*-chloro mercuribenzoic acid (*p*CMB) (a cystein proteases inhibitor).

To understand what kind of proteases make up the response to the increase in proteolytic activity in the different varieties of *Z. mays*. treated with citric acid, the H360 variety was tested using different inhibitors; PMSF (an inhibitor of serine proteases), pepstatin A (an aspartyl proteases inhibitor), EDTA (a metalloproteases inhibitor) and *p*-chloro mercuribenzoic acid (*p*CMB) (a cystein proteases inhibitor). The presence of serine protease was detected as well as aspartyl protease and there was a slight increase in metalloprotease activity, but no induction of cystein protease activity was detected (Figure 3).

Discussion

Various strategies for the elimination of pesticides have been suggested or used, such as transgenic plants with observed ethical and legal controversy in some countries; biocontrol with organisms that are difficult to control or manipulate; improvement of current pesticides (with less damage to the environment), and traditional strategy of improving resistant plants.

To reduce the irrational use of pesticides in vegetables production, alternative methods to control disease are required that would be economical effective and one such method is the "immunization" of plants against the pathogens with metabolites produced by the plants in different stress situation such as carboxylic acid. It is known that vegetable roots exude citric acid as one of the principles responses to well defined abiotic stress situation that include toxicity to metallic cations as Iron, Potassium, Phosphorus and

Oxygen deficiency (López Bucio et al., 2000; Marschner and Römheld, 1994). In plants, citric and other organic acid have a positive influence on the availability of nutrients in the soil and on the macrobiotic activity of the rhizosphere, as was observed in the availability and absorption of phosphorus by *Lupinus albus* L., *L. angustifolius* L., *L. leteus* L. and *Z. mays* (Jones and Darrah, 1995; Egle et al., 2003). However, it is not known if the citrate has other, different effects on the plant and we investigated the effect of foliar application of citric acid on the physiology of *Z. mays*, specifically in the proteolytic activity and soluble protein. Of the four hybrids evaluated, Falcon, HV313 and H360 showed positive results to the foliar application of citric acid in yield, with increase between 540 to 945 $kg \cdot ha^{-1}$. It was also observed that citric acid induced an increase of the measured biomass for soluble protein in the HV313 and H360 hybrids. The results are in agreement with the observation that 1 mM citric acid induced an increase in the protein of mitochondrial alternative oxidase (AOM) from the root of *Poa annua* and from cultures of cells tobacco in suspension. In both cases, there was no increase in enzymatic activity (Van Lerberghe and Mcintosh, 1997; Millenaar et al., 2002). But an increase in the dry weight of wild tobacco plants treated with citric acid, in the transgenic plants for over production and excretion of citric acid towards the rhizosphere (Lopez Bucio et al., 2000).

Proteolytic activity is ubiquitous in the biological system. In plants, proteolysis is a requirement for the mobilization of stored proteins of seeds used in germination, for the efficient recycling of amino acids in the senescence and apoptosis and into the housekeeping functions such as the activation of zymogens, the removal of aberrant proteins and protein degradation as part of a homeostatic cycle of protein removal and renovation.

The role of proteolytic activity has been well studied in regards to stress response in plants, for example, to hydric, saline and temperature stresses, wounds, treatment with ethylene, glucose deprivation, light and presence pathogens (Schaffer and Fischer, 1988; Rodrigo et al., 1991; Linthorst et al., 1993; Jones and Mullet, 1995; Jones et al., 1995). Citric acid induced a differential increase in the proteolytic activity in the *Z. mays* hybrids. The induction observed in proteolytic activity was found between the intervals of 2 to 12 times. The Falcon, HV313 and H360 hybrids responded better to foliate treatment with citric acid that Buffalo and Falcon hybrids. The PCA is a multivariate statistical method that is versatile and easy to use which was developed to extract the maximum amount of information from multidimensional dataset expressed as a matrix (Merdia et al., 1979; Drew et al., 1998). The H360, HV313 and Falcon hybrids responded to foliar treatment with citric acid and all three are recommended for commercial propagation in this condition. But, the Buffalo hybrid (B) responded weakly to foliar treatment with citric acid. The behavior of this variety is due to the fact of that genotype is of intermediate cycle where a longer vegetative cycle means better potential grain production. Although after the treatment with citric acid it was a slight increase in proteolytic activity and soluble protein.

The response in different varieties of *Z. mays* to citric acid foliar treatment was an increase in proteolytic activity. Where the presence of serine protease was detected as well as aspartyl protease and there was a slight increase in metalloprotease activity. This observation suggesting that proteolytic activity of some *Z. mays* hybrids is inducible by citric acid and it is part of plant defense response against deleterious abiotic factors that inducing the obtaining of more vigorous plants. It was reported in *Phaseolus vulgaris* a plant sensitive to water stress, that the aspartyl protease are involved in stress cause by lack of water. Nevertheless, in *Vigna unguiculata* a related specie to common bean and resistant to drought, the aspartyl protease showed a different stimulation (Cruz de Carvalho et al., 2001). Likewise, it is known in *Arabidopsis* that serine proteases are involved in heat shock and oxidative stress (Itzhaki et al., 1998). Was not detected any induction of cystein protease

activity by citric acid in corn hybrids, it cannot be ruled out whether these protease is increasing in the corn leaf treated with citric acid, since it is known that in peas and broccoli the cystein proteases are involved in drought and saline stress (Linthorst et al., 1993; Jones and Mullet 1995; Coupe et al., 2003). Alternatively, hemoglobin could be bad substrate for to measure the cystein protease activity inducted *de novo*.

We do not know if the increase in proteolytic activity is due to an increase in the enzymatic protein, as is the case with mitochondrial alternative oxidase of *Poa annua* and tobacco cells in suspension (Millenaar et al., 2002). It is possible that the citrate affects the protease inhibitors or induces enzymatic activation, or both. Studies to understand this role are currently underway.

Conclusions

The effect of citric acid on the dry weight and vigor of plants exposed to it is not just attributable to the effect produced on the modification of pH and the induction of macrobiotic activity of the rhizosphere or the capacity to form complexes with metallic ions or the mobilization of phosphorus. We have detected that citric acid has an effect in the physiology of the *Z. mays* leaves, especially in the increase of soluble protein and proteolytic activity. In the field in semi-arid climates, the propagation of the H360, Falcon and H313 varieties treated with citric acid are recommended. They can be applied to the crops before the appearance of the flag leaf to induce systemic resistance until the stage of the flowering of the corn.

Acknowledgements

This work was supported by the Universidad Michoacana de San Nicolas de Hidalgo (CIC-2.1MMP98 project).

References

Anson, M. L. 1938. The extinction of pepsin, tripsin papain and cathepsin with hemoglobin. J. Gen. Physiol. 22:79-86.

Bolan, N. S., R. Naidu, S. Mahimairaja and S. Baskaran. 1994. Influence of low molecular weight organic acids on the solubilization of phosphates. Biol. Fert. Soils 18:311-319.

Coupe, S. A., B. K. Sinclair, L. M. Watson and J. A. Heyes. 2003. Identification of dehydration-responsive cysteine proteases during post-harvest senescence of broccoli florets. J. Exp. Bot. 54:1045-1056.

Cruz de Carvalho, M. H., A. D´Arcy-Laeta, H. Roy-Macauley, M. Gareil, H. El Maaroul, A. T. Pham-Thi and Y. Zuily-Fodil. 2001. Aspartic protease in leaves of common bean (*Phaseolus vulgaris* L.) and Cowpea (*Vigna unguiculata* L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. FEBS Lett. 492:242-246.

Doubrava, N., R. Dean and J. Kuc´. 1988. Induction of systemic resistance to anthracnose caused by *Colletotrichum lagenarium* in cucumber by oxalate and extracts from spinach and rhubarb leaves. Physiol. Mol. Plant Pathol. 33:69-80.

Drew, M. G. B., G. R. H. Wilden, W. J. Spillane, R. M. Walsh, C. A. Ryder and J. M. Simmie. 1998. Quantitative structure activity relationship studies of sulfamates $RNHSO_3Na$. Distinction between sweet, sweet-bitter and bitter molecules. J. Agric. Food Chem. 46:3016-3026.

Egle, K., W. Römer and H. Keller. 2003. Exudation of low molecular weight organic acids by *Lupinus albus* L., *Lupinus angustifolius* L. and *Lupinus luteus* L. as affected by phosphorus supply. Agronomie 23:511-518.

Gorlach, J., S. Vorlach, G. Knauf-Beiter, G. Hengy, U. Beckhove, K. H. Koegel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann and J. Ryals. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8:629-643.

Itzhaki, H., L. Naveh, M. Lindahl, M. Cook and Z. Adam. 1998. Identification and characterization of DegP, a serin protease

associated with the luminal side of the thylakoide membrane. J. Biol. Chem. 27:7094-7098.

Jones, D. L. and P. R. Darrah. 1995. Influx and efflux of organic acid across the soil root interface of *Zea mays* L. and its implications in rhizosphere C flow. Plant Soil 173:103-109.

Jones, J. T. and J. E. Mullet. 1995. A salt and dehydratation inducible pea gene, *Cy15a*, encode a cell wall protein with sequence similarity to cystein proteases. Plant Mol. Biol. 28:1055-1065.

Jones, M. L., P. B. Larsen and W. R. Woodson. 1995. Ethylene regulated expression of a carnation cysteine proteinase during flower petal senescent. Plant Mol. Biol. 28:505-512.

Kraffczyk, I., G. Tolldenier and H. Beringer 1984. Soluble root exudates of maize: Influence of potassium supply and rhizosphere microorganisms. Soil Biol. Biochem. 16:315-322.

Kuc´ J. 1999. Plant immunization. A non-pesticide control of plant disease. Petria 1:79-83.

Kuc´, J. and S. Tuzun. 1990. Metabolic regulation of resistance genes in tobacco for the control of blue mold. In: C. Main and H. Spurr, (Eds.) p. 35-46. Blue mold disease of tobacco literature service, North Carolina State University, Raleigh.

Linthorst, H. J., C. Van Der Does, F. T. Brederode and J. F. Bol. 1993. Circadian expression and induction by wounding of tobacco genes for cysteine proteinase. Plant Mol. Biol. 21:685-694.

Lopez Bucio, J., M. F. Nieto Jacobo, V. Ramirez Rodriguez and L. Herrera Estrella. 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 160:1-13.

Lowry, D. H., N. J. Rosenbrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:268-275.

Lyons, G. D., T. Reglinski and A. C. Newton. 1995. Novel disease control compounds: the potential to immunize plants against infection. Plant Pathol. 41:407-427.

Ma, J. F., S. J. Zhenf, H. Matzumoto and S. Hiradate. 1997. Detoxifying aluminum with buckwheat. Nature 390:569-570.

Marschner, H. and V. Römheld. 1994. Strategies of plants for acquisition of iron. Plant Soil 165:265-274,

Merdia, K. V., J. T. Ken and J. M. Bibby. 1979. Multivariate analysis. Academic Press, London, pp. 213-254.

Millenaar, F. F., A. Gonzalez Meler, J. N. Siedow, A. M. Wagner and H. Lambers. 2002. Role of sugar and organic acids in regulating the concentration and activity of the alternative oxidase in *Poa annua* roots. J. Exp. Bot. 53:1081-1088.

Mitchell, R. E. and D. R. Walters. 1995. Systemic protection in barley against powdery mildew infection using methyl jasmonate. Asp. Appl. Biol. 42:251-256.

Pellet, D. M., D. L. Papernik and L. V. Kochien. 1996. Multiple aluminum resistance mechanisms in wheat: Roles of roots apical phosphate and malaic exudation. Plant Physiol. 112:591-597.

Reglinski, T., P. R. Poole, G. Whitaker and S. M. Hoyte. 1997. Induced resistance against *Sclerotinia sclerotorium* in kiwi fruit leaves. Plant Pathol. 46:716-721.

Rodrigo, I., P. Vera, L. C. Van Loon and Conejero, V. 1991. Degradation of tobacco pathogenesis-related proteins. Evidence for conserved mechanism of degradation of pathogenesis-related proteins in plants. Plant Physiol. 95:616-622.

Ryan, P. R., E. Delhaize and P. J. Randall. 1995. Characterization of Al-stimulated efflux of malaic from apices of Al-tolerant wheat roots. Planta 196:103-110.

Salt, S., S. Tuzun and J. Kuc´. 1986. Effects of ionone and abscisic acid on the growth to tobacco and resistance to blue mould. Physiol. Mol. Plant Pathol. 79:1152-1160.

Schaffer, M. A. and R. L. Fischer. 1988. Analysis of mRNAs that accumulate in response to low temperature identifies a thiol protease gene in tomato. Plant Physiol. 87:431-436.

Toal, E. S. and P. W. Jones. 1999. Induction of systemic resistance to *Sclerotinia sclerotiorum* by oxalic acid in oilseed rape. Plant Pathol. 48:759-767.

Van Lerberghe, G. C. and L. Mcintosh. 1997. Alternative oxidase: from gene to function. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48:703-734.

Yang, Z. M., M. Sivaguru, W. J. Horn and H. Matzumoto. 2000. Aluminum tolerance is achieved by exudation of citric acid from roots soybeans (*Glycine max*). Physiol. Plant. 110:72-77.