# EFFECT OF CITRIC ACID ON THE PROTEOLYTIC ACTIVITY OF *Zea mays* L.

## Efeito do ácido cítrico sobre a atividade proteolítica de *Zea mays* L.

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**ABSTRACT**

Hybrids of *Zea mays* L. (Buffalo, Falcon, H360 y HV313) were treated with citric acid (2000 ppm). Grain yield, soluble protein and proteolytic activity were monitored when the crop reached physiological maturity. Citric acid was applied before the appearance of the flag leaf, and induced an increase in the grain yield from 4222 to 5780 kg/ha, in the soluble protein from 6.34 to 7.91 mg/mg and into the proteolytic activity from 14.3 to 65.7 µU mg prot$^{-1}$. This is an increase of 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 differentially induces proteolytic activity and vigor in the corn hybrids analyzed.

**Index terms:** Maize, citric acid, proteolytic activity.

**RESUMO**

*Zea mays* L. híbridos (Buffalo, Falcão, H360 e HV313) foram tratados com ácido cítrico (2000 ppm). O rendimento de grãos, proteína solúvel e atividade proteolítica foram monitorados na fase de maturação fisiológica do cultivo. O ácido cítrico aplicado antes do aparecimento da folha bandeira induziu um aumento na produção de grãos 4222 a 5780 kg/ha, na proteína solúvel de 6.34 – 7.91 mg/mg de peso seco e atividade proteolítica de 14.3 to 65.7 µU mg prot$^{-1}$, esto é, um incremento de 2-12 vezes nos híbridos Falcao, H360 e HV313, enquanto o híbrido Buffalo responderam com menor intensidade ao tratamento com ácido cítrico. No híbrido H360, tratadas com ácido cítrico, apresentou-se um aumento na atividade proteolítica de aspartil proteases e serin protease. Os resultados indicam que o ácido cítrico diferencialmente induz a atividade proteolítica e o vigor de híbridos de milho testados.

**Termos para indexação**: Milho, atividade proteolítica, ácido cítrico.

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## INTRODUCTION

The growing demand for agricultural products produced without pesticides has motivated the development of alternative strategies for controlling disease and pathogens to increase crop yield (LYONS et al., 1995; KUC´, 1999). Pathogen attacks may occur during the early stages of crop development, and heavy damage sustained here strongly correlates with plant stress levels, both in the flowering and the development of the grain.

Inducing the plant to acquire systemic resistance to pathogens may be a viable alternative strategy. Systemic resistance can be achieved with the application of metabolites during the plant's development, forcing a defensive response, for example, the exogenous application of methyl jasmonate in barley crops, or benzothiadiasole and 2,6-dichloroisonicotinic acids on wheat crops (MITCHELL; 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´; TUZUN, 1990). Molecules derived from pathogens such as glucans have been applied successfully to diverse vegetable crops as well. Doubrava et al. (1988) demonstrated that oxalic acid and carboxylic 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; 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 tobacco plants. Also, the 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 *carboxilic* acids such as citric acid in plant responses to environmental stress is complex and is just 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

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(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 an increase in the vigor of the plant (MA et al., 1997; PELLET et al., 1996; RYAN et al., 1995; 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 its effect on the vigor of the plant has been well studied. Nevertheless, it is not known if citrates have other effects on the plant, apart from those already described. The aim of this study was to investigate whether the grain yield, protein soluble and proteolytic activity of several commercial corn hybrids can be induced when citric acid is applied to their leaves. We can report that citric acid led to an accumulation of soluble protein in the leaf and a differential induction of proteolytic activity in the 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 101º 01´ longitude at an average altitude of 1,828 masl. The area has an average annual rainfall of 600 mm with vertisol terrain that contains over 70 % clay, and has a pH that varies from 7.8 to 8.8. The soil has 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, an early cycle hybrid with a period of 138–150 days from planting to physiological maturity; average height 240 cm with 64–77 days till flowering; of white grain with high protein content and good industrial use quality that has moderate resistance to stem and cob rotting.

• Buffalo, an intermediate cycle hybrid with a period of 152–163 days from planting to physiological maturity; average height of 235 cm with 78–89 days till flowering; is resistant to stem and cob rotting.

• HV313, a varietal hybrid with an early-intermediate cycle with a period for 145–155 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, an intermediate cycle trilineal hybrid generated with 75% tropical germplasm and 25% subtropical germoplasm of white grain; with a period of 158–163 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. When they reached physiological maturity, six plants from each of the four corn hybrids were collected at random for processing in the laboratory. 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 stored at 4º C during the collecting. From fragments of the vegetable organs asepsis was achieved with a mixture of: 15 % extran; 70 % ethanol; 3 % hydrogen peroxide and 0.5 % sodium hypochlorite. After the asepsis process they were washed exhaustively with sterile deionized water and stored in plastic bags at a temperature of -70º C until their use.

### Proteolytic activity assay

This was carried out 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 % polyvinylpolypyrrolidone ($v \cdot v^{-1}$). 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, 37º C), and is reported as U µg $prot^{-1}$.

### Inhibition of proteolytic activity

The following inhibitors were used; 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 using the Lowry method (LOWRY et al., 1951).

### Data analysis

The 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. Evaluation of the statistical significance of the parameter was based on the Student *t* test ($p$ = 0.5). Information about grain production, proteolytic activity and soluble protein (taken as an indicator of mass) of the corn hybrids was analyzed by Principal Component Analysis (PCA) to determine 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 groups are similar, PCA will identify the top position amongst them.

## RESULTS AND DISCUSSION

The yields, proteolytic activity and soluble proteins of the Falcon, Buffalo, HV313 and H360 hybrids were evaluated. The Buffalo hybrid presented the poorest yields, with results being similar to plants that had not received 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·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 increase was modest for the Buffalo hybrid, intermediate in HV313 and H360 while the highest increase was in Falcon (Table 1). The observed increase in proteolyic activity was found to be from 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). Due to the fact that all the hybrids used gave a positive response and differential to the treatment with citric acid, a principal component analysis (PCA) was used to determine which of them gave the best response. The first PCA describes a total variance of 71.01 % for the three variables shown in Table 1. In Figure 2, three distinct groups are shown. The first group contains the control hybrids (without citric acid) whilst 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 group shows that citric acid did not induce any effect on proteolytic activity.

To understand what kind of proteases make up the response to the increase in proteolytic activity in the different varieties of *Z. mays* L. 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).

Various strategies for the elimination of pesticides have been suggested or used, such as transgenic plants, provoking 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 a traditional strategy of improving resistant plants.

To reduce the irrational use of pesticides in vegetable production, alternative methods to control

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 |

disease are required that would be economically effective. One such method is the "immunization" of plants against pathogens with metabolites produced by the plants in different stress situations, such as carboxylic acid. It is known that vegetable roots exude citric acid as one of the principles responses to well defined abiotic stress situations that include toxicity to metallic cations as Iron, Potassium, Phosphorus and Oxygen deficiency (LÓPEZ BUCIO et al., 2000; MARSCHNER; RÖMHELD, 1994). In plants, citric and other organic acids have a positive

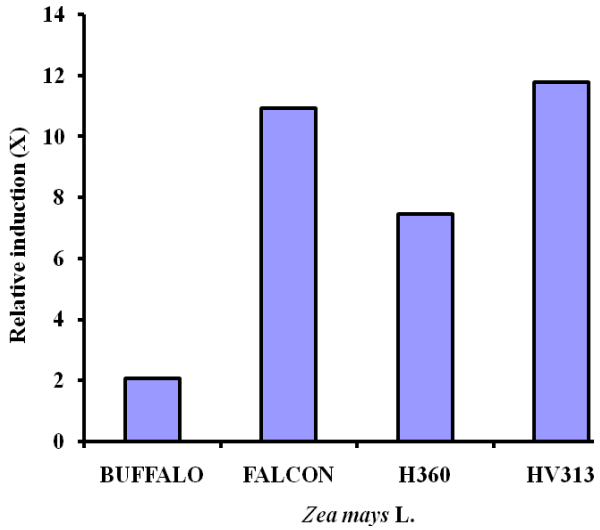


Figure 1 – Relative induction of the proteolytic activity of *Z. mays* hybrids induced with citric acid. Proteolytic activity data from Table 1 was taken to show the difference in responses between maize hybrids treated with and without citric acid.

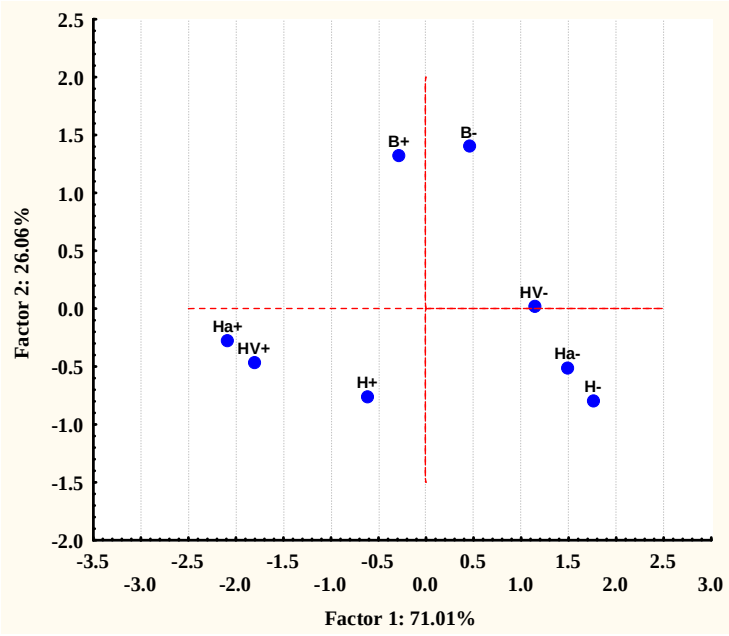


Figure 2 – Principal component analysis of grain production, proteolytic activity and soluble protein in leaves of *Z. mays* hybrids treated with citric acid. Maize hybrids, Ha (Falcon), H (H360) and B (Buffalo). (-) Control and (+) treatment with citric acid.

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* L. (EGLE et al., 2003; JONES; DARRAH, 1995). However, it is not know if the citrates have other, different effects on the plant and we investigated the effect of foliar application of citric acid on the physiology of *Z. mays* L., 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/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 (MILLENAAR et al., 2002; VANLERBERGHE; MCINTOSH, 1997). However, an increase in dry weight has been observed where wild tobacco plants have been treated with citric acid, and also in transgenic plants engineered for the 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 (JONES; MULLET, 1995; JONES et al., 1995; LINTHORST et al., 1993; RODRIGO et al., 1991; SCHAFFER; FISCHER, 1988). Citric acid induced a differential increase in the proteolytic activity in the *Z. mays* hybrids. The induction observed in proteolytic activity was found to be from 2 to 12 times. The Falcon, HV313 and H360 hybrids responded better to foliate treatment with citric acid than Buffalo and Falcon hybrids. PCA is a multivariate statistical method that is versatile and easy to use which was developed to extract the

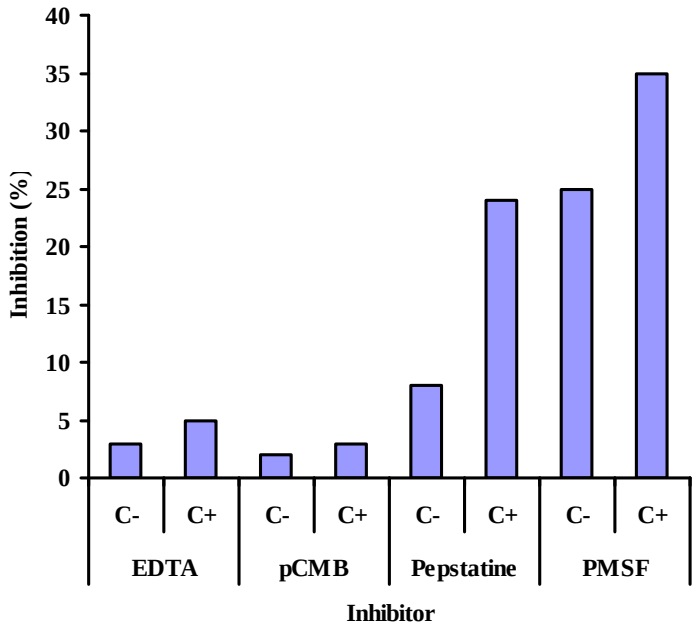


Figure 3 – Effect of class specific protease inhibitor in the proteolytic activity of *Z. mays* L. Extracts from leaves of the hybrid H360 were used and the proteolytic activity was measured in the presence of 0.2 mM PMSF (an inhibitor of serine proteases), 0.15 mM pepstatin A (an aspartyl proteases inhibitor), 10 mM EDTA (a metalloproteases inhibitor) and 2 mM *p*-chloro mercuribenzoic acid (*p*CMB) (a cystein proteases inhibitor). (C-) = without and (C+) = with citric acid.

maximum amount of information from multidimensional datasets expressed as a matrix (DREW et al., 1998; MERDIA et al., 1979). 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 that the genotype is of intermediate cycle where a longer vegetative cycle means better potential grain production. However, after the treatment with citric acid there was a slight increase in proteolytic activity and soluble protein.

The varieties of *Z. mays* responded to the citric acid foliar treatment with an increase in proteolytic activity. Where the presence of serine protease was detected as well as aspartyl protease there was a slight increase in metalloprotease activity. This observation suggests that proteolytic activity of some *Z. mays* hybrids is inducible by citric acid, thus by inducing the plants' defense response against deleterious abiotic factors it is possible to obtain more vigorous plants. It was reported in *Phaseolus vulgaris*, a plant sensitive to water stress, that aspartyl protease are involved in stress caused by lack of water. Nevertheless, in *Vigna unguiculata*, a related species to the 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). There was no induction of cystein protease activity detected by applying citric acid to the corn hybrids. However, it cannot be ruled out whether these proteases are 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 (COUPE et al., 2003; JONES; MULLET, 1995; LINTHORST et al., 1993). Alternatively, it could be that hemoglobin is a bad substrate in which 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 these interactions are currently underway.

## CONCLUSION

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 on the physiology of *Z. mays* leaves, especially in the increase of soluble protein and proteolytic activity. In semi-arid climates, the propagation of H360, Falcon and H313 varieties treated with citric acid is recommended. Citric acid 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.

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