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Full Length Research Paper

Chitosan application in maize (*Zea mays*) to counteract the effects of abiotic stress at seedling level

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Worldwide, the conditions of biotic and abiotic stresses adversely affect the potential production of maize. Drought or heat facilitate the infection with fungi such as *Aspergillus flavus* and *Fusarium moniliforme*, and consequently increase the production of mycotoxins. There are several strategies for managing the problem, but in the future, people will prefer the cleaner and cheaper technology. The use of elicitors for protection of corn can be considered a cheap and clean technology. Chitosan elicitor is a linear polysaccharide produced commercially by deacetylation of chitin. It has been reported that this elicitor induce phytoalexin accumulation in plant tissue. Application of chitosan to seeds in rice significantly increased rice yield. About this, there are no reports in corn. For this reason, the aim of this study was to determine the protective effect of chitosan in maize seedlings subjected to abiotic stresses. To this end, three treatments were tested (a negative control, a positive control, and a group coated with chitosan solution) under four abiotic stresses conditions since their germination stage: drought, moisture, acid pH and alkaline pH. During five weeks, the seedlings growth was evaluated by measuring their total length, the length of leaves, stems and the thickness of these and presence of fungi. Positive effect was observed in seeds treated with chitosan or stressed with acidic pH in dimensions of seedlings and there was no fungal growth.

Key words: Abiotic stress, *Zea mays*, chitosan, pH, drought, humidity.

INTRODUCTION

Maize (*Zea mays*) is one of the most important cereals in the world because it is the staple food in many low-income countries. Worldwide, 159,531,007 ha are harvested per year; however, productivity is often diminished by some external factors that exert a negative influence on the cereal growth (FAOSTAT, 2009). One of these factors is the presence of abiotic stress, namely, all climatic and environmental conditions that affect the optimal crop growth like temperature, soil pH modifications and extreme humidity or drought, which cause seeds damage since cultivation (Laffite, 2001; Boyer and Westgate, 2004; Tester and Bacic, 2005). Drought reduces maize yields annually up to 15% in lowland, tropical and subtropical regions, causing estimated losses that reach 16 million tons of grain (FAOSTAT, 2009). Otherwise, if watering is excessive, the plant may rot resulting in the formation of sludge that stores water for several days without renewing, creating pollution conditions and excessive moisture , which is exploited by opportunistic microorganisms (particularly the fungi *Aspergillus flavus* and *Fusarium moniliforme*) which are able to colonize the plant roots consuming nutrients that crops require, so the plant reduces its growth allowing

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pathogens to parasite it, producing irreversible damages (Widstrom, 1996; Placinta et al., 1999).

The pH is generally used as the key chemical diagnostic parameter in the evaluation of soil and water. For example, with an acidic pH, some metal ions present in the soil become toxic, while with a slightly alkaline pH, problems of low solubility of ions or high levels of sodium and metal sulfides can be found. The optimum pH of maize growth ranges between 5.5 and 7.5, which allows the corn planting in a wide variety of soils, but when these pH values cannot be achieved, tolerant crops should be chosen in order to fit in the best in soil acidity conditions (Magnavaca and Bahia, 1993; Aguirre, 2001).

Although plant breeders have used genetic variation of maize to select cultivars with improved performance under specific environmental stresses (Laffite, 2001; FAO, 2003; Bhatnagar-Mathur, 2008), there are other alternatives for achieving the optimal growth of crops at adverse conditions, such is the case of the use of elicitors, which are molecules that induce plant defense mechanisms by the perception and transduction of biological signals to activate defense responses (Radman et al., 2003; Angelova et al., 2006).

One of this molecules is chitosan (CH), an oligosaccharide and a biopolymer found in the cell walls of some fungi, but whose main source of production is the chitin hydrolysis in an alkaline medium at high temperatures (Devlieghere et al., 2004; Muzzarelli and Muzzarelli, 2005; El Hadrami et al., 2010). It is known that chitosan has antimicrobial properties, therefore, it is a powerful biocide and a novel elicitor satisfactorily employed in preserving fruits, vegetables, and recently in seeds, because there has been found a favourable reduction in the amount of pathogenic fungi in stored grains and seeds treated with it, and it has been demonstrated that the seeds germination capability is major when CH is used (Bhaskara et al., 1999; Miranda, 2000a; Devlieghere et al., 2004; Muzzarelli and Muzzarelli, 2005, Agrawal et al., 2002; Guan et al., 2009). However, despite the virtues of chitosan as a preservative in seeds, there are few published studies related to the application of this biopolymer in corn crops under abiotic stresses (Ruan and Xue, 2002; Shao et al., 2005; Guan et al., 2009). The aim of this study was to evaluate the effect of a chitosan coating on maize seeds under abiotic stress through measuring the physical aspects of seedlings.

To carry out this investigation, we evaluated *in vivo*, during five weeks and under greenhouse conditions, the effect of chitosan as a coating on corn seeds on the germination and growth (thickness of stems, total length of the leaves and stems) of seedlings under abiotic stress conditions. After the fifth week of growing, it was evaluated qualitatively (*in vitro*) the potential effect of chitosan on the leaves of maize seedlings during their growth, to determine if the crop triggers defense mechanisms against fungal development, resulting from biotic stress caused by the imposition of acid and alkaline pH to soil, as well as drought and moisture conditions present in farmland, which is a serious problem worldwide due to the high levels of pollutants and rainfall fluctuations that have occurred in recent years.

MATERIALS AND METHODS

Materials

QPM (High Quality Protein Maize, (Krivanek et al., 2007) seeds (LM8M L-8) of the Maize Breeding Program from the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) based in the Campo Experimental del Bajío, México (with a moisture content of 11.7% and a germination rate of 100%), were used. For the seeds coating, chitosan obtained from chitin shrimp exoskeletons, with a molecular weight of 1836.277 g/mol and a deacetylation degree of 95% was used, and it was produced and characterized in our laboratories without further purification (Miranda, 2000b).

Coating preparation

A 2% chitosan solution (CH) was prepared, dissolving 10 g of the biopolymer in 500 ml of water acidified with acetic acid (Meyer Chemical SA de CV, Tlahuac, DF, Mexico), keeping under constant agitation for 24 h and adjusting the pH to 5.0 with a 12% sodium hydroxide solution (JT Baker, Xalostoc, Edo. de Mexico, Mexico).

Seeds coating

Seeds were soaked in the 2% chitosan solution for 5 min and then they were dried in an oven at 29°C for 24 h to suppress excessive moisture given by the biopolymer solution.

Establishment of abiotic stress conditions

Ten seeds per pot were sown. In order to establish the abiotic stress conditions in maize seeds, the pots were divided into four groups: In the first group, the ideal soil irrigation conditions were modified to simulate drought conditions (ISTA, 1993), while in the second group, humidity conditions were simulated (ISTA, 1993). Moreover, a third group of pots was manipulated by changing the pH of the soil acidifying it with a 0.1% phosphoric acid solution (JT Baker, Xalostoc, Edo. De Mexico, Mexico); thus decreased the pH to 5.30. Finally, the farmland of the fourth group was alkalinized simulating a calcareous soil by adding 0.02 M calcium carbonate (JT Baker, Xalostoc, Edo. De Mexico, Mexico), achieving an increase of pH to 8.00. Each of these four groups were divided into three subgroups: a negative control where seeds were not subjected to stress and no coating was applied; a positive control where seeds were stressed but not covered, and a treatment in which seeds were stressed and coated with the chitosan solution (Treatment 1).

This study was conducted in the greenhouse of the Grains and Seeds Research Unit in Facultad de Estudios Superiores, Cuautitlán, UNAM, which was previously sanitized in order to carry out the planting under protected conditions (ISTA, 1993; Albajes et al., 1999; Sonneveld and Voogt, 2009) to avoid interference in the experimentation results.

Evaluation of maize seeds germination

All seeds were sown at the same time. Germination was evaluated after the fifth day of sowing.

Evaluation of maize seedlings growing

This assessment was carried out for five weeks through the observation of the general physical aspects of the plant and the weekly measurements of the number of sprouts, leaf length, stem length, thickness of stems and the total length of the seedling.

Presence of maize specific biotic agents in leaves, caused by the induction of abiotic stress in soil

In the fifth week of growth, small pieces of leaf seedlings were cut and washed with a 0.01% sodium hypochlorite solution (Meyer Chemical SA de CV, Tlahuac, DF, Mexico). Then they were placed by triplicate in Petri dishes with Potato Dextrose Agar (MCD Lab, Tlalnepantla Estado de Mexico, Mexico). Finally they were then incubated at 28°C during 8 days in order to observe the specific fungal growth of the corn crop (*A. flavus* and *F. moniliforme*).

Data analysis

The evaluations of *in vivo* studies conducted in the greenhouse were evaluated by repeated measurements ANOVA with a Post Hoc Turkey test using the software Statistica Release 7 (StatSoft, Inc., USA). Mean values with statistical difference of $p < 0.05$ were considered as significant.

RESULTS

In general, it can be emphasized that there was no effect of chitosan in the phenological variables of maize seedlings, while, with regard to stress factors, the acidic pH was the one who had a significant effect over the phenological variables, reflected in a further growth of the seedling structures. By the other hand, chitosan treatment had a positive effect on microbiological development in the plant, acting as a seed protector against maize pathogenic fungi under abiotic stress conditions. In order to facilitate the presentation of this information, all aspects of measuring will thus be shown separately.

In most cases, the seeds germination percentage was of 100%. The exception was the negative control subjected to drought, which germination rate was 90%. In all the cases, sprouts began to appear at the fifth day of planting with normal characteristics. Subsequently, we present the qualitative and quantitative features of seedlings structures according to stress and treatment applied.

Changes in soil pH

Leaves

Stressed seedlings by changes in farmland pH were significantly shorter than those corresponding to the negative control during the 4th and 5th week (data not shown). CH treatment did not favour a major leaf length, but, while untreated alkalinized seedlings presented chlorotic spots and necrosis after 15 days of growing, leaves from coated seeds did not present sickness symptoms.

Stems

As thickness is concerned, stems of seedlings grown in alkaline soil were significantly thinner than those subjected to acid stress. CH treatment did not increase stems length. From the third week of growing, it was shown a higher growth of seedling stems subjected to acid pH stress, and there was a statistically significant difference ($p=0.0765$) when compared with the seedlings subjected to alkaline pH.

Seedlings

Qualitatively, seedlings under acidic conditions and CH cover remained green, vigorous and devoid of disease. Control seedlings under alkaline conditions showed weakness, yellowing, chlorosis and wilting, but the ones treated with CH became greener in the third week of growing. During the first two weeks of growth, seedling lengths were not affected by pH soil conditions, but from the third week, acidic plants were significantly larger than the alkaline ones, exceeding them up to 20 cm (Figure 1).

Changes in irrigation conditions

Leaves

All leaves from stressed plants showed a dry green colour, even those with CH treatment. In the fifth week of growth, leaves subjected to drought conditions began to fade rapidly.

Stems

From the third week of growth, seedlings stems with irrigation shortages acquired a dark coloration, but they had greater lengths than those under humidity conditions; however, from the fourth week, stems length was the same for both stresses. As thickness is concerned, humidity conditions caused the growth of significantly thinner stems than the rest of the seedlings during weeks two and three; however, in the 5th week, they began to thicken equalling the thickness to those submitted to drought stress. CH treatment produced thicker stems regardless of stress during the first three weeks; however, at the 4th week, this trend was halted and the stems thickness was overtaken by the negative control group (data not shown).

Seedlings

Seedlings grown under drought conditions showed better

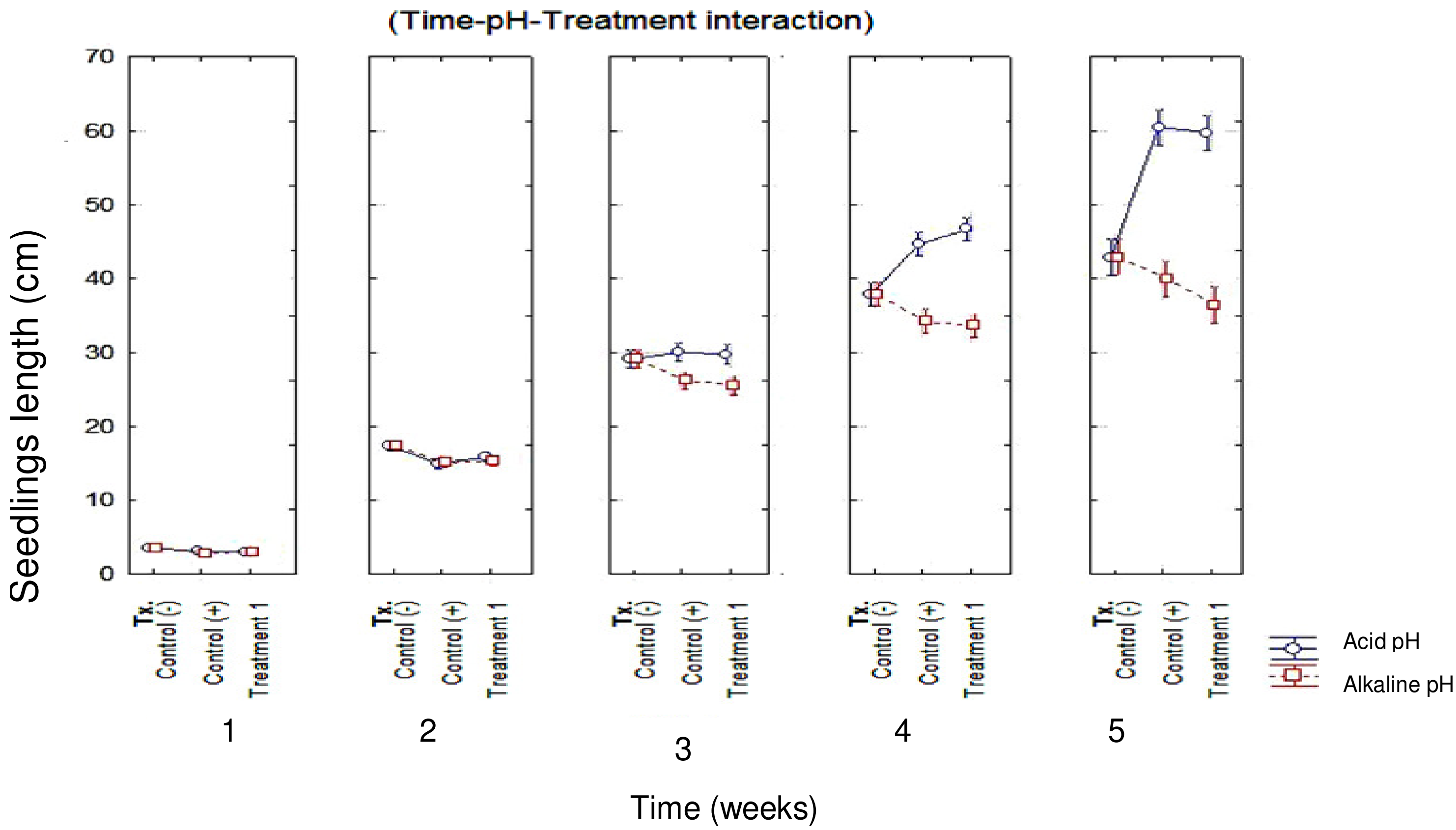


Figure 1. Length of seedlings stressed by changes in the pH of arable land (p = 0,00000). Control (-) corresponds to seeds without stress or treatment, Control (+) are stressed seeds, and Treatment 1 are stressed and coated maize seeds.

features than those grown under excessive wet conditions during the first two weeks. Total seedlings lengths were similar during the first two weeks, but in the fifth week, there was a difference of about 10 cm between the total length of the seedlings without stress and those stressed regardless of treatment (Figure 2). From the fourth week, seedlings subjected to drought slowed their growth, while those subjected to humidity continued their normal development. In this case, the addition of CH to the seed was not a useful treatment to achieve the quantitative increase in the stressed seedlings length.

Presence of maize specific biotic agents in leaves, caused by the induction of abiotic stress in soil

Because there is scientific evidence that development of fungal species on crops and seeds is associated with weather and soil acidity (Payne et al., 1988; Laffite, 2001; Cotty and Jaime-García, 2007), seedlings obtained from abiotic stressed seeds were studied to determine the correlation between abiotic factors and the presence of the fungus *A. flavus* and *F. moniliforme*.

Table 1 show that the acidic pH per se retards the fungal growth, because while the negative control presented an advanced fungal burden, the positive control is almost starting to develop fungi. Although, some bacteria were manifested in the acidic CH treated group, there was no evidence of *A. flavus* and *F. moniliforme* in that plaque. By the other hand, it can be seen that CH is an effective treatment against fungal burden in maize seedlings under alkaline pH conditions, because while the controls presented an abundant fungal growth (and in the positive control even other fungal species development), in the plaque with treated samples, only bacteria could grow.

According to Table 2, although, it seems that drought conditions favoured the development of both fungi and bacteria (positive control), CH treated plants showed no fungal development. Otherwise, humidity conditions favoured the development of fungi and bacteria, and under these irrigation conditions, CH does not act as an inhibitor of fungal growth.

DISCUSSION

According to our results, there were no differences between germination rates of CH treated seeds and their corresponding controls. This behaviour is counter to what

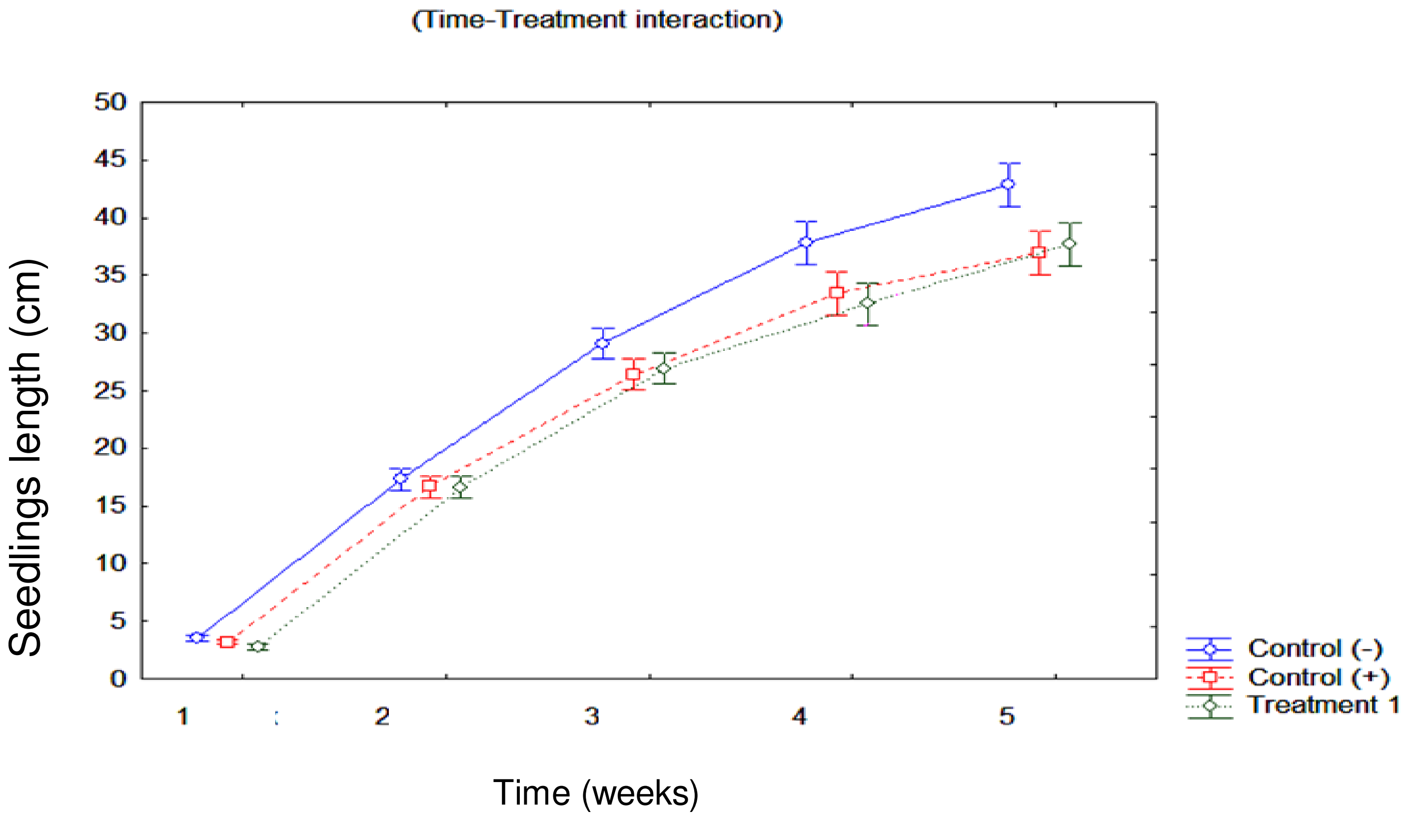


Figure 2. Length of seedlings stressed by changes in irrigation conditions (p = 0.01430). Control (-) corresponds to seeds without stress and treatment, control (+) are stressed seeds, and Treatment 1 are stressed and coated maize seeds.

Table 1. Qualitative analysis of microbial presence in maize leaves from stressed seedlings by changes in pH soil by the fifth week of growing.

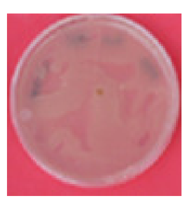
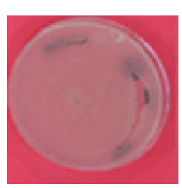
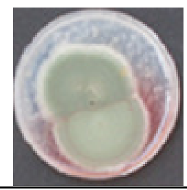
Group	**Abiotic stress (pH)**	
	Acid pH	**Alkaline pH**
Control (-) (no covering, no stress)	Presence of bacteria. Growth of *A. flavus* and *F. moliliforme* in an abundant way all around the Petri dish (*F. moniliforme* is evidenced with a cottony white colour in the Petri dish base. *A. flavus* is evidenced by a sporulated greenish spiral coming from the middle of the Petri dish.	
Control (+) (no covering but stressed)	Abundant bacterial burden. Little fungal *F. moliliforme* colonies beginning to grow over the leaf sample. 	Fungal growth of different species: *A. flavus F. moliliforme* and *Penicillium sp.* Mild bacterial burden. 
Treatment1 (stressed, CH covered)	Bacterial growth all around the agar plaque, but null presence of fungal burden. 	Abundant bacterial growth all around the agar plaque, but null presence of fungal burden. 

Table 2. Qualitative analysis of microbial presence in maize leaves from stressed seedlings by changes in soil irrigation conditions by the fifth week of growing.

Group	Abiotic stress (irrigation condition)			
	Drought		**Humidity**	
Control (-) (no covering, no stress)	Presence of bacteria. Growth of *A. flavus* and *F. moliliforme* in an abundant way all around the petri dish (*F. moniliforme* is evidenced with a cottony white colour in the petri dish base. *A. flavus* is evidenced by a sporulated greenish spiral coming from the middle of the petri dish.			
Control (+) (no covering but stressed)	Important bacterial growth beginning to develop. *F. moliliforme* and *A. flavus* colonies grow around the sample.		Mild bacteria. Abundant growth of *A. flavus* and *F. moliliforme* with visible spores.	
Treatment1 (stressed, CH covered)	Abundant bacterial growth all around the agar plaque, but null presence of fungal burden.		Abundant growth of *A. flavus* and *F. moliliforme* with lots of visible spores present in the whole agar plaque. Few bacteria.	

was reported by Reddy et al. (1999), because they found an increase in germination rates of wheat seeds treated with CH, while Guan et. al. (2009) reported a minor germination time in seeds subjected to low temperature abiotic stress and previously treated with CH.

There are few published studies concerning the use of defence mechanisms inducers against abiotic stress with good results (Ruan and Xue, 2002; Shao et al., 2005; Guan et al., 2009) because most of the researchers are devoted to combat these factors and are focused on the creation of crops with intrinsic resistance to this stress (Edmeades et al., 1992; Laffite, 2001; Herrera and Martínez, 2004). Such investigations are based on traditional breeding techniques to create genetically modified plants, such as those engineered to produce an excess of metabolites in their structures for better tolerance to toxic metals in acid soils. Nevertheless, researches have been done using elicitors in seeds to combat abiotic stresses on crops, getting enhanced germination indexes, increased shoot heights, root lengths, and shoot and root dry weights in the case of maize lines and accelerating germination time and improving tolerance to stress conditions in the case of rice (Ruan and Xue, 2002; Boonlertnirun et al., 2008; Guan et al., 2009).

As to plant illness is concerned, Reddy et al. (1999), found an increase in wheat seed resistance to certain common diseases when applying CH to seed before planting. Equally, CH has also been extensively utilized as a seed treatment to control *F. oxysporum* in many host species, diminishing the plant damage significantly, as well as it has been used specifically in wheat seeds to induce resistance to *F. graminearum*, and therefore, improving the seed quality (Bhaskara et al., 1999; Rabbea, 2003). These results are consistent with ours, because we demonstrated that application of CH in the seed prior its germination confers the plant some immunity to pathogens, because most of the times, plants grow healthier than the untreated ones and free of fungal pathogens despite of the abiotic stress to whose they were subjected, and moreover, seedling sickness is combated by the reduction of the inoculum quantity given by CH.

Regarding to the presence of the fungi *A. flavus* and *F. moniliforme* in plants under pH stress, it could be observed that in those crops where the pH was changed, the presence of chitosan retarded the development of these fungi. This behaviour is based on that chitosan is an activator of some enzymes involved in plant defence against pathogens, such as glucanase, chitinase and chitosanase, which are activated once the pathogen reaches the host, by what enzymes are able to identify the components of cell wall and begin to degrade pathogen affecting its growth and development in the crop, being an effective method of fighting against it without affecting the normal plant growing (Lineart et al., 1983; Shao et al., 2005).

It could be seen that the proper acidification of soil allowed maize seeds to germinate and grow with healthy physical features. By those conditions, seedlings structures grew in an accelerated way, and when adding CH to the seed, it did not develop fungal burden. This suggests that this seeds are suitable for cultivation in mountainous soils where acidic soils are common. In this particular case, the outcome of chitosan as a fungal protector as seen in Table 1, is probably due to the optimal interaction with phosphoric acid, allowing a greater bioavailability of certain nutrients and the presence of phosphorus in soil. These finding contradict many authors who argue that soil acidity prevents the optimal growing of maize crops without previous seed treatments (Aguirre, 2001; Laffite 2001). Otherwise, application of CH in alkaline seedlings promote the fungi development, this may be because CH as a cover in the seed did not dissolve while watering, becoming unavailable to cross the cell seeds membrane and thence, unavailable to protect the seed by activating signalling cascades.

It is important to note that seeds per se are contaminated with *A. flavus* and *F. moniliforme* (as shown in the negative control of Tables 1 and 2), confirming that they live naturally in grains and seeds. When stressing the seeds with changes in irrigation conditions, it could be seen that humidity is an important factor in fungal growth, because in the case of excessive watering, *A. flavus* and *F. moniliforme* appeared abundantly in the Petri dishes, generating a great quantity of spores (Control (+)). This indicates that those fungal species require high levels of humidity to grow in maize crops. It can be because water rots roots turning them weak, so, those microorganisms are able to parasitize the plant systemically from roots to leaves; that's why they can be catalogued as facultative parasites (according to the definition given by Agrios, 2005). As for the positive control, seedlings from seeds subjected to humidity, but previously treated with CH (Treatment 1) presented a plenty growth of the studied fungi, meaning that the biopolymer under abundant watering conditions, does not act as a fungal seed protector. This coating inactivity could be given because chitosan, in its molecular structure has an amino group (NH_2) that makes it hygroscopic, and when getting in contact with water, the amino group is protonated turning into an ammonia group (NH_3), which confers more hygroscopicity to the CH molecule, so, the seed traps the farmland moisture, allowing the seed rotting, and it can be parasite easily by *A. flavus* and *F. moniliforme* as described before.

On the other hand, some authors have found that reduction of drought stress by irrigation reduces aflatoxin contamination in corn. Drought tolerant corn varieties were found to produce significantly less aflatoxins in the field under drought conditions compared to aflatoxin-resistant controls. It also has been reported that contamination of maize with *A. flavus* is exacerbated by late season drought stress (Chen et al., 2004; Fountain el al., 2007). It supports the results obtained when stressing seeds with limited irrigation, because as it was presented in Table 2, at a seedling level, there was few presence of fungal burden in the agar plaques, because at that stage, fungi are not capable to develop because they do not have the nutrients and environmental conditions they need to develop. For the same reason, seedlings from stressed and CH treated seeds did not develop fungal burden, because the biopolymer acted as an inhibitor in association with the drought stress conditions which avoid the *A. flavus* and *F. moniliforme* growing at young seedling level.

CONCLUSION

The use of chitosan as a protective coating on maize seeds under abiotic stresses allows seedlings to develop defence mechanisms against the fungi *A. flavus* and *F. moniliforme*, which is manifested in the decrease or absence of disease in the different structures of the seedling. The acidification of farmland to pH ~ 5, allows QPM maize seeds to germinate properly promoting a further growth of seedlings with long, thick and strong stems, leaves and roots; in addition, these conditions coupled with the chitosan coating, allow crops to grow free of disease. Although more research is still needed to understand the defence mechanisms activated by chitosan, this promises to be a biotechnological alternative for crops growth disease-free from protected seeds, where products would be free of sickness from farm to consumption, decreasing losses caused by adverse climatic and environmental conditions.

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