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IMPROVEMENT OF COTTON SEED QUALITY BY BROTOMAX

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Abstract

Cotton seed germination and root development in seedlings is promoted in the presence of Brotomax (up to 0.1% v/v). Ten days after planting, increased taproot and lateral root growth was observed compared to controls. Thirty-day-old Brotomax-treated seedlings exhibited increased root development with lateral roots more enlarged than that seen in control seedlings. Accompanying this effect, the chlorophyll content of leaves from Brotomax-treated plants (2.7 mg/g fw) was higher than that in leaves from control plants (2 mg/g fw). Moreover, the phenolic content of the leaves and stems of treated plants (0.98 mg/100 g fw and 1.04 mg/ 100 g fw, respectively) was higher than that measured in the leaves and stems of control plants (0.89 mg/100 g fw and 0.97 mg/100 g fw, respectively).

Twenty-five-day-old seedlings infected with *Fusarium oxysporum* and treated with Brotomax showed increased total chlorophyll content and lower phenolic compound content than similarly infected but untreated control plants. Moreover, in *F. oxysporum*-infected seedlings, treatment with Brotomax reduced the level of ethylene production compared to that seen in control seedlings. Finally, PCR experiments showed that the advance of *F. oxysporum* in seedlings treated with Brotomax was lower than that observed in control seedlings. These results demonstrate that Brotomax increases cotton seedling tolerance to infection by vascular fungi such as *F. oxysporum*.

Introduction

Enhanced emergence together with increased root system growth at low soil temperature is very important in protecting cotton seedlings exposed to adverse environmental conditions, which can increase seedling susceptibility to pathogen activity (Mc Michael, 1998). As such, timely, consistent and vigorous cotton seedling establishment is critical to establishing yield potential in cotton production (Becker, 1997).

The germination and development of cotton seedlings is conditioned by both biotic and abiotic factors. Among the biotic factors, numerous pathogens such as *Fusarium* spp., *Verticillium dahliae*, *Rhizoctonia solani*, *Pythium* spp. and *Thielaviopsis basicola* are implicated in seedling diseases which affect cotton seed germination, roots or lower stem properties. The effects of such fungal diseases, coupled with edaphic factors such as excessively high soil water content, soil crusting, or extreme soil compaction, may impede plant growth and development. Acute symptoms associated with these diseases in seedlings give rise to stand losses, reduced uniformity of the stand and reduced seedling establishment due to impeded growth of the taproot and lateral roots of the plant. Such effects necessitate replanting, with associated delays in crop development and reduction of crop yield (Becker, W.D. and Hopper, N.W., 1998).

Application of fungicides in the pre- and post-emergence stages of seedling development reduces the extent of pathogen activity. However, continued use of these fungicides may result in pathogen resistance, soil contamination or contamination of food products destined for animal or human consumption.

To this extent, the stimulation of factors that contribute to the natural resistance of cotton plants to fungal pathogens provides an alternative to the use of traditional fungicides. For example, some investigations have shown that Brotomax, a liquid fertilizer, increases the compounds implicated in plant defense mechanisms such as levels of phenolic and terpenic compounds, reduces plant wilt by decreasing vascular occlusion, and enhances tolerance to different fungal pathogens (Botía et al., 2001).

The present study was conducted to evaluate the effects of Brotomax on cotton seed germination, on the vigour and emergence of seedlings, and on levels of chlorophyll, phenolic compounds, and ethylene production in seedlings. Furthermore, the influence of Brotomax on the advance of *F. oxysporum* in inoculated cotton seedlings was assessed.

Materials and Methods

Plant Material, Seeds, Brotomax Treatment and Measurement of Seed Germination and Root Growth

Cotton seeds (var. *Delta Opalo* (Delta Opal)) were supplied by Deltapine (Sevilla, Spain). The seeds were soaked for 6 h in water (control) or in an aqueous solution of Brotomax (0.03, 0.09, 0.15, 0.2, 0.3, 0.5 and 1%, for treated). After soaking, the seeds were germinated in Petri dishes containing water or respective aqueous solutions of Brotomax. After 10 days, the percentage germination of cotton seeds and root length in these plants was assessed.

Plant Culture Conditions, Brotomax Treatment and Inoculation with Fungus

Cotton seeds (var. *Delta Opalo*) were germinated in trays with vermiculite in a growth chamber at 25 ºC with a 16-h photoperiod. The plants were divided randomly into two lots, the first of which (control group) was watered regularly, and the other with an aqueous solution of Brotomax (0.1%). Fifteen days after planting, each lot was further divided into two similar groups. One group was inoculated with *F. oxysporum* and the other group was used as control (uninoculated plants). Inoculation with fungus was realized by an incision in the bottom of the stem.

Measurement of Chlorophyll and Phenolic Compound Content

Control and Brotomax-(0.1%) treated plants (cultivated on vermiculite in trays) were collected at days 15 (non-infected (control)) and 25 (10 days after fungus inoculation). For chlorophyll extraction, plant leaves were homogenized in acetone 80% (10mg/ml) and levels of chlorophyll a, b and total chlorophyll were determined by spectrophotometric analysis at wavelengths of 645 and 663 nm. For the extraction of phenolic compounds, different parts of plants were harvested (roots, stems and leaves). These were ground up and then shaken with dimethylsulphoxide (DMSO) (100 mg fresh weight/ml) for 1 h for extraction. The corresponding extracts were filtered through a 0.45 μm nylon membrane before spectrophotometric analysis using a UNICAM UV/VIS spectrometer UV2 (Unicam Limited, Cambridge, UK) to estimate total phenol content. This was expressed as gallic acid/100 g FW according to the Folin Ciocalteu Method (Singleton and Rossi, 1965).

In Vitro Plant Culture and Measurement of Ethylene

Cotton seeds var. *Delta Opalo* were sterilised by soaking in 100% ethanol for 1 min and then in a 20% v/v Domestos^{R} solution for 15 min. They were rinsed several times in sterile water before being implanted on a 0.7% w/v agar medium (control) or this same medium supplemented with 0.1% Brotomax. Thirty days after germination (plant height about 7 cm with first leaves evident), infection with *F. oxysporum* was realized by an incision in the lower stem. In each case, similar lots of uninoculated plants were used as controls. For the measurement of ethylene, 1ml gas samples were withdrawn at different times and measured in a HP 5890 Series II gas chromatograph equipped with a flame ionization detector and a stainless steel column (3m length, 1/8” inner diameter) filled with alumina. Ethylene concentration was calculated from a calibration plot of known concentrations of standard ethylene samples.

PCR Analysis

The *Fusarium oxysporum* f. sp. *vasinfectum* DNA isolation and PCR methods used were the described by Abd-Elsalam et al, 2003.

Chemicals

The standard phenolic compound (gallic acid) and Folin Ciocalteu's phenol reagent were purchased from Sigma (St. Louis, MO, USA). Brotomax was supplied by Agrometodos, S.A (Madrid, Spain).

Results and Discussion

Effect of Brotomax on Seed Germination and Root Growth in Stress Conditions

Cotton seeds were soaked in water or aqueous Brotomax solutions as described in the Material and Methods section and then germinated in Petri dishes. Exposure to high water content during the germination phase probably contributed to the low percentages of seed germination that were measured, these being as low as about 30% under control conditions (Fig. 1). Such conditions may occur “in vivo” when germination and growth is realized in waterlogged soil. Treatment with aqueous solutions of Brotomax was found to improve germination rates and development of the root system (Fig. 1, insert). Of the different Brotomax dosages tested, the maximum dosage (0.1%) gave the best result, with a seed germination rate of 40% and a 26% increase in taproot length compared to untreated control plants.

Influence of Brotomax on Root Development

The stimulating effect of Brotomax on growth of the root system is evident from Photo 1, which shows enhanced lateral root (root hair) development in 10-day-old Brotomax-treated seedlings (Photo 1A). Thirty-day-old Brotomax-treated plants also showed a more highly developed root system, with thicker lateral roots compared to untreated control plants (Photo 1B).

Some reports exist in the literature suggesting that host morphology acts as a determinant of resistance, although a general unsubstantiated view exists that the absence of vascular pathogens in gymnosperms is related to the short length of tracheid elements. Rudolph (1968) claimed that the resistance of hop cultivars to *Verticillium albo-atrum* was a function of the point of emergence of the first lateral root from the tip. Cultivars in which this interval was short were more tolerant to the disease. Phillip and Wilhelm (1971) showed a similar correlation in cotton, which is probably related to disease escape. In this way, *Gossypium barbadense* cv. Waukena White produced more lateral roots deep in the soil and was less susceptible to *V. dahliae* than *G. hirsutum* cv. SJ-1 with fewer deep laterals. While the obvious explanation that deep-originating lateral roots may escape the bulk of soil inoculum may be true, such a comparison in two highly polygenic *species* is not strictly valid when other resistance features in these plants are taken into account.

Influence of Brotomax Treatment on the Chlorophyll and Phenolic Compound Content of Cotton Plants

Once an optimum Brotomax concentration (0.1%) was identified, which showed an improved seed germination and root system development after seed treatment, we sought to analyze the effect of such a dose on plants cultivated on vermiculite. This was done by assessing the effects of 0.1% Brotomax on photosynthetic activity (as measured by chlorophyll a, b and total content) and the self-defense capacity of seedlings against fungal infection (as evidenced by phenolic-phitoalexin compound content). An increase of approximately 35% in the chlorophyll a, b and total chlorophyll content of leaves of 15-day-old Brotomax-treated plants was found compared to untreated control plants (Fig.2A). Furthermore, a higher level of phenolic compounds (about 10%, Fig.2B) was measured in the stem and leaves of Brotomax-treated plants compared to untreated control plants, but the difference was not significant between in relation to the roots of control and treated plants.

For 25 day-old Brotomax-treated plants, a 20% increase in the total chlorophyll content and a 15% increase in the phenolic compound content of the leaves of these plants was observed compared to control (Fig. 3 A and C). No significant differences, however, were observed between control and treated plants in relation to the phenolic compound content of the root.

Ten days after inoculation with *F. oxysporum*, Brotomax-treated (0.1%) plants showed an increased chlorophyll a, b and total chlorophyll content (a total increment of 18%) compared to control plants (Fig. 3C). Furthermore, inoculation with *F. oxsporum* altered the total phenolic compound content of Brotomax-treated and untreated plants (Fig. 3D). In this way, increments of 60, 76 and 52% were observed for roots, leaves and stems, respectively in untreated plants, compared to a 40% increment in Brotomax-treated plants.

Ethylene Production

F. oxysporum-inoculated cotton plants cultivated “in vitro” on control medium or on medium supplemented with Brotomax (0.1%) exhibited high levels ethylene production at the time of initial infection. This can be explained by the injury-induced stress caused to plants (Fig. 4). The ethylene content then fell to undetectable levels in control plants infected with *F. oxysporum* (Fig. 4), but from the third day post-inoculation these plants showed increased ethylene production which could be attributed to the activity of the fungus. Such an effect gives rise to an increased stomatal aperture in leaves and therefore higher rates of transpiration, resulting in a greater demand for water, or desiccation in a water-deficient environment. In infected plants cultivated in a 0.1% Brotomax-supplemented medium, the fungal activity still resulted in a rise in ethylene production, but the measured levels were lower than those recorded from plants cultivated under control conditions.

Ethylene has been shown to play a multi-faceted and major role in wilt diseases (Pegg 1976, 1981). Wiese and DeVay (1969, 1970) showed a strong correlation between *Verticillium dahliae* evolved ethylene, host ethylene and defoliation of cotton. Ethylene from T9 (defoliating strain)-infected cotton increased fivefold, 13 days after inoculation, whereas only a twofold increase occurred with SS4 (non-defoliating) infection. The essential direct and indirect involvement of ethylene in other aspects of *Verticillium* pathogenesis has been described by DeVay (1989) and Pegg (1989). The link between pectolytic enzymes and ethylene is complex (Pegg, 1981), involving de novo synthesis of enzymic mRNA (Bennet and Della Penna, 1987) and increased tissue sensitivity to the enzyme (Cronshaw and Pegg, 1976).

Ethylene production has been seen as a function of loss of leaf turgor in Fusarium wilt (Pegg and Cronshaw (1976). Epinasty, adventitious root production, chlorosis, necrosis, tylosis, supernumerary xylem formation and patterns of stomatal opening can all be attributed to increased ethylene levels (Misaghi et al., 1969). Mussell et al. (1982) further describe an enzyme capable of generating ethylene from 1-amino cyclopropane carboxylic acid (ACC), liberated from tomato cell walls by a purified polygalacturonase from *Verticillium dahliae*. The ethylene-producing reaction mixture was stimulated by IAA, Mn^{2+} and p-coumaric acid. Ethylene production was inhibited in the presence of a competitive inhibitor of IAA oxidase (phenolic compounds). The case for ethylene involvement is strong since the process can be reversed by the application of the ethylene inhibitor, silver thiosulphate. The results of Van der Molen et al. (1983) suggest that vascular blockage occurs as a result of changes to the primary cell wall by depolymerase enzymes induced by host ethylene. The available evidence suggests that ethylene may be both a cause and a result of pectolytic activity in a complex synergism (Abeles, 1973).

Effect of Brotomax on *F. oxysporum* Advance in Cotton Seedlings

The stimulating effect of Brotomax on phenolic content, chlorophyll content and ethylene production has direct repercussions on the advance of *F. oxysporum* in seedlings. That is, in 15-day-old seedlings the advance of *F. oxysporum* was 2 cm in the direction of the roots and 1 cm along the stem in control seedlings, whereas in Brotomax-treated plants *F. oxysporum* had advanced only 1 cm in the direction of the roots (Fig. 5). These results demonstrate that Brotomax increase the natural defense mechanism of cotton plants, thereby impeding the advance of *F. oxysporum* into the plant.

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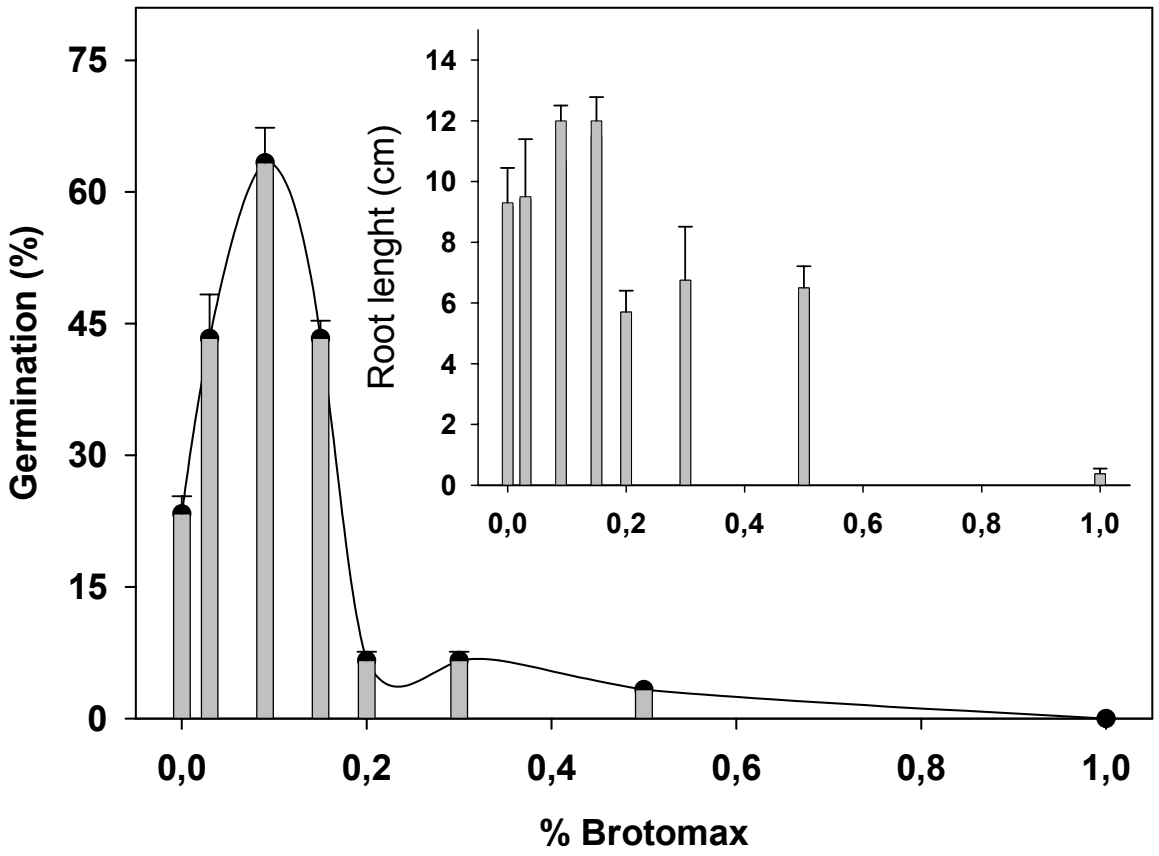

Figure 1. Effect of Brotomax on germination (%) and root length (cm) in cotton seeds (var. *Delta Opalo*. Data correspond to mean values in control and Brotomax-treated seeds (0.03, 0.09, 0.15, 0.2, 0.3, 0.5 and 1%). Vertical bars denote ± SE (n=3).

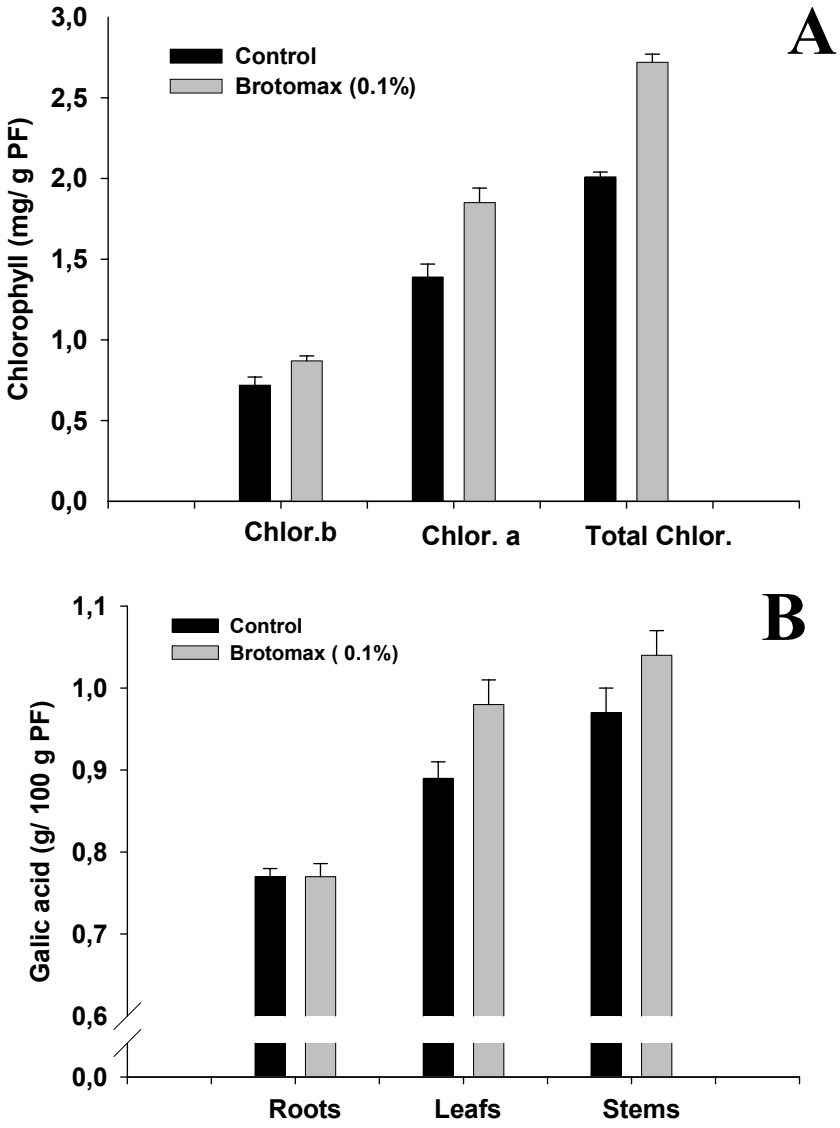

Figure 2. Effect of Brotomax on chlorophyll (A) and total phenol (B) levels in different components of cotton plant (var. *Delta Opalo*). Data correspond to mean values: (A) a, b, total chlorophyll (mg/g FW), (B) gallic acid (g/100 g FW) in control and 0.1% Brotomax-treated plants 15 days after germination. Vertical bars denote ± SE (n=3).

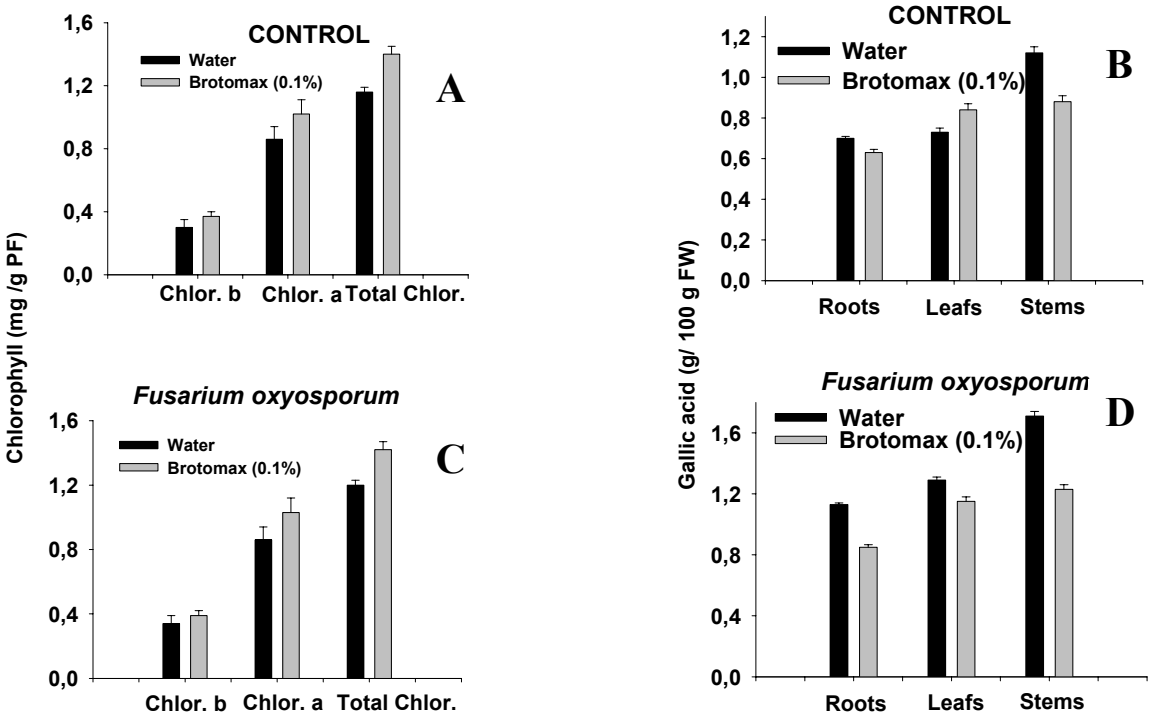

Figure 3. Effect of Brotomax on chlorophyll (A and C) and total phenol (B and D) levels in different components of cotton plant (var. *Delta Opalo*) infectaed with *Fusarium oxysporum* sp. *vasinfectum*. Data correspond to mean values: (A and C) a, b, total chlorophyll (mg/g FW), (B and D) gallic acid (g/100 g FW) in control and 0.1% Brotomax-treated plants 25 days after germination (ie. 10 days after fungal inoculation). Vertical bars denote ± SE (n=3).

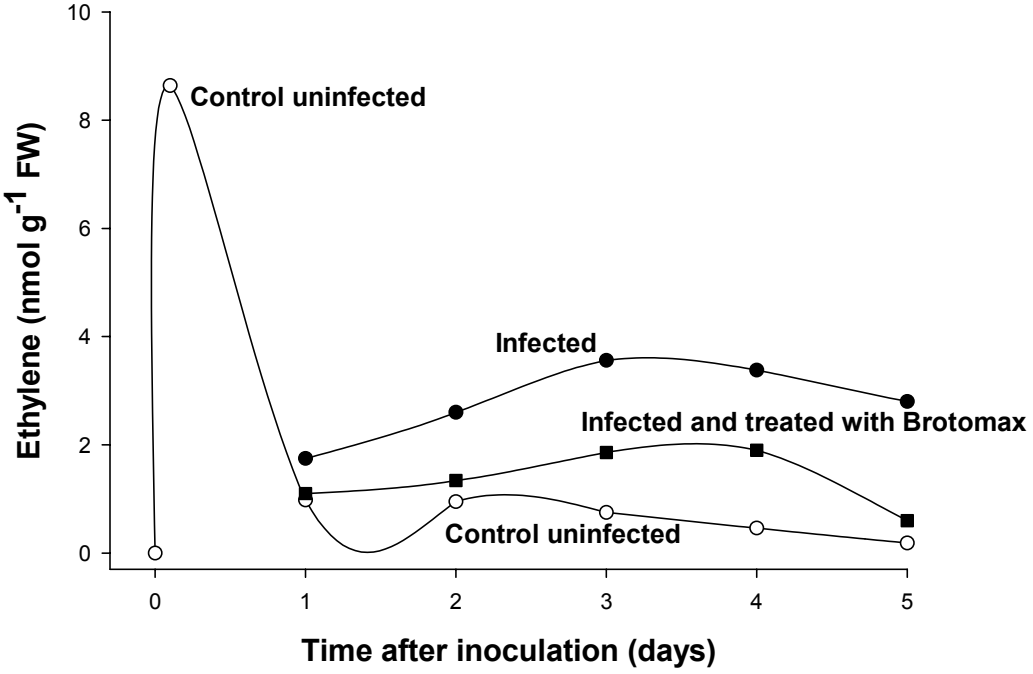

Figure 4. Effect of inoculation with *F. oxysporum* on ethylene production *in vitro* in cotton plants. Plants were grown on a 0.7% w/v agar media (controls) and in the same culture media to which an aqueous solution of Brotomax had been added at a final concentration of 0.1%. The inoculation with fungus was carried out on 30-day-old plants. Ethylene production (nmol g^{-1} FW) in these plants was determined at different times post-inoculation. The experiment was repeated in ten plants, with typical data from one of them shown here.

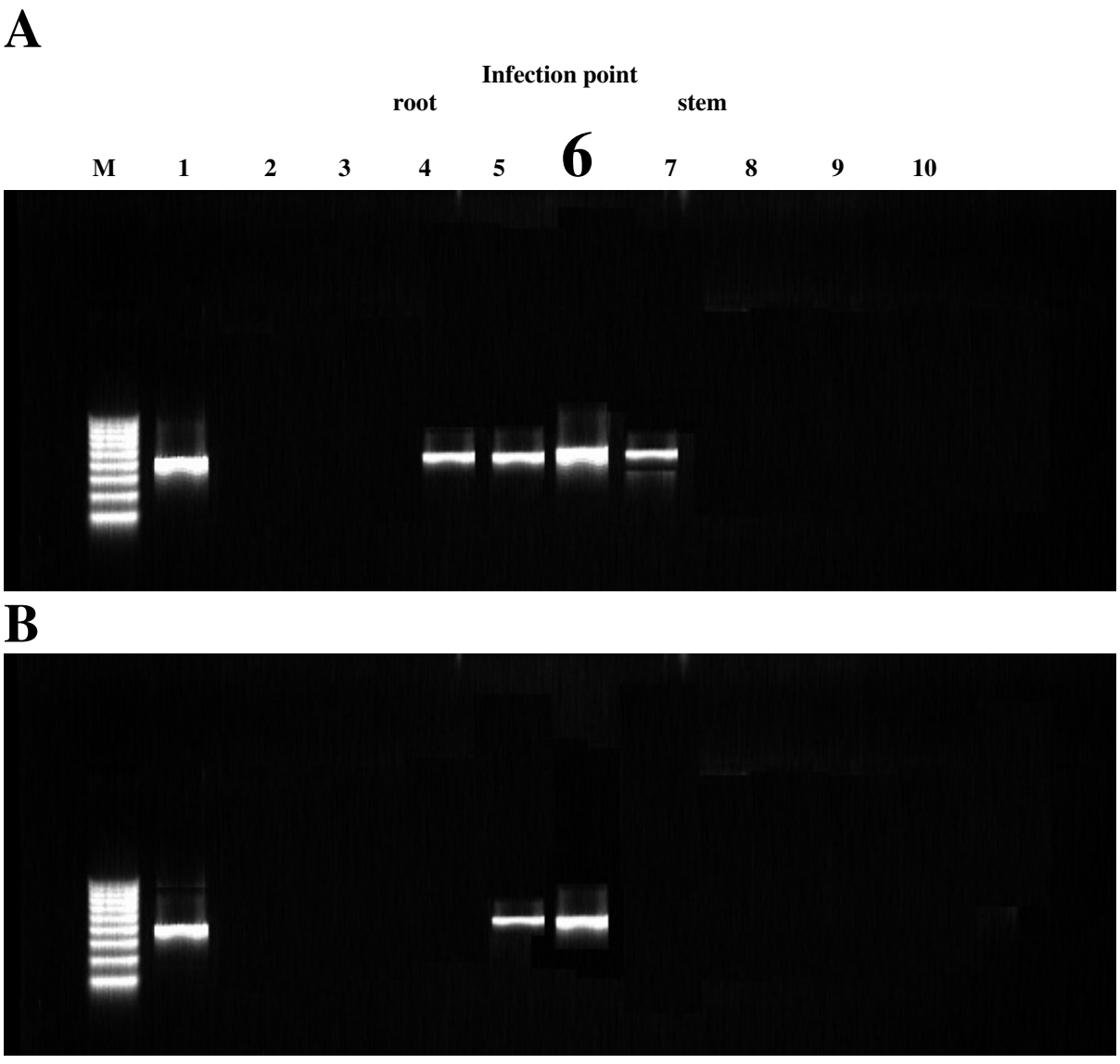

Figure 5. Full-ITS PCR products amplified for *F. oxysporum* with ITS-Fu-f and ITS-Fu-r primers. Each lane corresponds to 1 cm section of stem or root of cotton plant infected with *F. oxysporium*. A) Control plants. B) Plants treated with Brotomax. M: molecular-weight markers; lane 1 DNA amplified from mycelium *Fusarium oxysporum*; lanes 2-5 DNA amplified from cotton roots; lanes 6-10 DNA amplified from cotton stems.

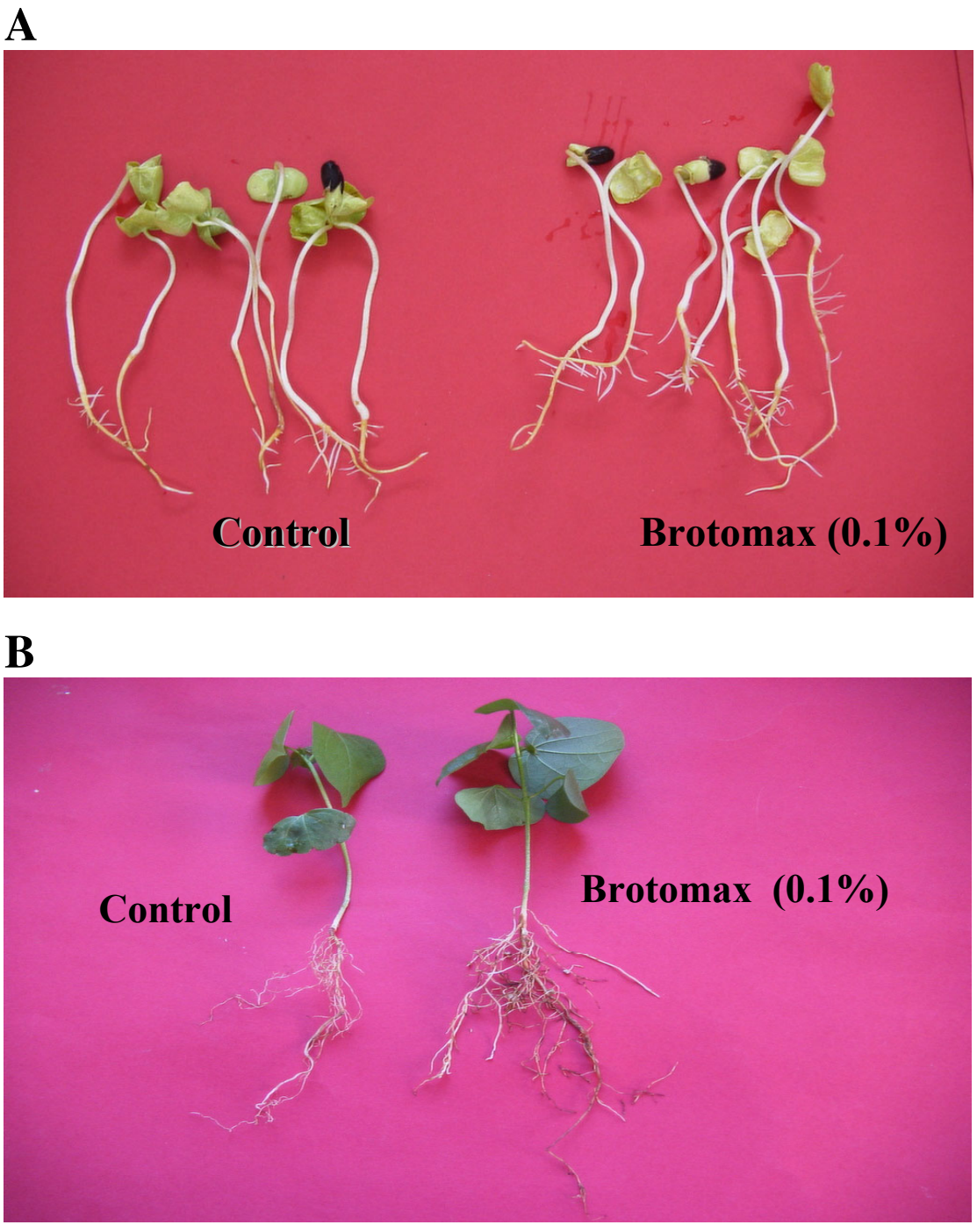

Photo 1. Effect of Brotomax on root development. A) 10-day-old cotton seedlings germinated in dark in a Petri dish. B) 30-day-old seedlings grown in vermiculite under a 16-h photoperiod.