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INCREASE OF THE TOLERANCE AGAINST *VERTICILLIUM* WILT IN COTTON WITH BROTOMAX

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Abstract

The general pattern of symptoms of Verticillim wilt in cotton is a total or partial loss of turgor, due to development of a vascular occlusion. This obstruction is due to the formation of tyloses, which are formed by expansion of xylem parenchyma cells. These tyloses are formed in the xylem wall and grow to invade the vessel lumen of the roots and stem. Another reason for the decreased water flow in the xylem is due to the development of gels and gums, which are spore-trapping mechanisms that delay or prevent fungal spread. The developments of these structures is due to the presence of mycelia fungi within the xylem and colonize the plant by mycelia growth or by conidial production, traveling with the flow of water due to transpiration and germination in the stem xylem. This fungus invades different xylem through the pits from one vessel to another. Cotton plants treated with Brotomax increase their total content of phenolic compounds and gossypol (and its derivatives) in relationship to control plants, and the extract of this plants have high antifungal capacity or resistance against *Verticillium dahliae*. This increase of antifungal capacity was higher in stem and roots of cotton plants treated. The increases in the antifungal activity of cotton extracts in plants treated with Brotomax reduce the vessel occlusion. Cotton plants treated with Brotomax showed a mean increase of 773.04 $Kg.ha^{-1}$ (18.65%), although this data depended on the variety. For example, the increase for Carmen was 337.37 $Kg.ha^{-1}$ (8.83%) and for Crema 1078.11 $Kg.ha^{-1}$ (28.28%). Tolerance was also observed to depend on variety, with a mean increase of 0.24 (7.18%), the lowest value being for Carmen 0 (0%) and the highest for La Chata 0.50 (23.07%). These results show that varieties susceptible to disease have a higher increase in visual tolerance, although increase yield due to treatment with Brotomax does not depend on the genetic tolerance of the variety. The increase in yield and tolerance to Verticillium was significant at 0.01%, with a value of correlation of 0.56, which suggest that the tolerance visual is a good index of the production for all the varieties assayed. The differences between varieties and the repetitions was also significant but not between the variety and treatment. These results show that Brotomax can increase cotton yield and tolerance against Verticillium in the different varieties assayed.

Introduction

Verticillium wilt is a disease caused by a soil-borne fungus, *Verticilliun dahliae*. It was first reported in cotton by Carpenter (1914) in Virginia, and has since been reported from most countries and all continents where cotton is cultivated. The annual damage caused by Verticillium wilt amounts to 1.5 million bales worldwide (Bell, 1992), losses that affect many countries including Australia, Greece, Peru, South Africa, Spain, Syria, Turkey, and Zimbabwe and some states of Argentina, Brazil, India, and Mexico. However, such losses have been reduced, with regard to earlier decades, by the elimination of cultivars with low tolerance and the development of new tolerant varieties with improved agronomic performance and a integrated management system (appropriate management of nitrogen fertilizers, irrigation and others influential factors) (El-Zik, 1985). In spite of intense efforts to control this disease, these losses persist and much still needs to be done to improve the resistance of cotton to Verticillium wilt.

In Spain, Verticillium wilt has been known since the 1950´s. It was detected in Andalusia in 1972 (Jimenez-Diaz et al., 1974), although farmers had observed symptoms of the disease for several years prior to this report. In 1983, the disease was identified a defoliant pathotype in Las Marismas del Guadalquivir (south Spain) which later extended through most of the Guadalquivir Valley (south Spain) (Bejarano Alcazar, 1998). At the present time, most cotton fields of in Andalusia, where more than 90% of national cotton production is localized, show symptoms of this disease (RAEA, 2002).

The symptoms caused by Verticillium are the premature senescence of leaves, which leads to defoliation and the loss of photosynthetic area. The first noticeable symptoms are reduced plant growth, leaf epinasty (wilting), and a change in leaf color. Irregular chlorotic (yellow) patches begin to form between the main veins and along the margins of infected leaves. As these patches enlarge, tissue located in the centre of these areas die, giving the leaf a mottled appearance. Severely injured leaves may abscise.

The stage of plant development when foliar symptoms of Verticillium wilt occur has a direct effect on cotton lint yield and quality (Pullman and DeVay, 1982). If the disease occurs early, about 60 days after planting, it reduces total dry matter accumulation, internodes elongation and fruiting branch development; it increases shedding of square and small bolls and inhibits further production. Large bolls usually remain attached to the infected plant and eventually open, even after defoliation. Lint yield reductions are small when foliar symptoms appear after mid-August.

In Spain, when the symptoms appear early (Photo 1), the consequences can be devastating. Usually, those are expressed at two times: in July if temperatures are low and in September when temperature begin to decrease with shorter days and longer nights.

The bases of resistance towards *V. dahliae* are based on the following topics: reduced inoculum potential in the rhizosphere or rhizoplane, host physical restriction and limitation of the pathogen in the host, host chemical inhibition of the pathogen. When root and stems are infected with *V. dahliae*, the xylem tissues respond by synthesizing both antibiotic terpenoids (phytoalexins) and antibiotic flavonoids (tannins) (Bell et al., 1992 and 1993). The most resistant cultivar to Verticillium and Fusarium known, Seabrook Sea Island 12B2 (SBSI) of *Gossypium barbadense*, begins the rapid synthesis of both terpenoid phytoalexins and tannins in infected stele tissues 24-48 h earlier than susceptible or tolerant cultivars of *G. hirsutum*, confining the fungus in infected vessels before it can penetrate adjoining uninfected vessels and form new conidia to continue the systemic invasion of the xylem (Bell, 1994).

In this sense, Brotomax plays an important role by modulating of the biosynthesis of some phenolic compounds that increase the antifungal capacity of different species of agricultural interest, such as citrus (Fuster et al., 1994; Botía et al., 1995, 1996, 1997; Ortuño, 1997), olive (Del Río et al., 1998), vine and cotton. So, increased polyphenol levels resulting from treatment with Brotomax lead to improved self-defense and, depending on the time of application, help plants and trees to increase their yields, to induce greater vegetative growth and increase the chlorophyll content (in tomato, melon, tobacco and clover, for example).

The objective of this research was to ascertain the effect of Brotomax on different cotton cultivars as an inducer of self-defense, studying rises in yield, visual tolerance (plant index), changes in the levels of total phenolic compounds and gossypol and their derivates, and the increased antifungal capacity against *Verticillium dahliae* in extracts of cotton plants.

Materials and Methods

The field experiment were assayed in Las Cabezas de San Juan (Sevilla, south Spain), in a soil with a history of infestation. The varieties used were: Carmen, Delta Opalo (Delta Opal), Corona (DP-20), Crema (Stoneville KC-311), La Chata (Cocker) and Conchita, and their characteristics are described in Table 1.

Planting was carried out on 18 April with a density of 15-20 plants per lineal meter.

The experiments received the agronomic treatment characteristic of commercial crops in the locality.

Plants were harvest by hand at two times, the first on 10 October and the second six days later (16 October).

Treatments with Brotomax were realized at three different moments (3 June, 18 July and 23 August) with one concentration (1%).

The control and treated cultivars were grown in a split-plot design with three replications. The main plot consisted of a single row 10 m long and the distance between rows was 95 cm. The characters studied were determined in 5 m of each row.

To detect the effect of treatment with Brotomax, and its interaction with cultivars, an analysis of variance (ANOVA) and a study of the difference between treated and control plants were made.

Characteristics Studied

Infestation symptoms were studied from the plant index, which was made during 10, 11 and 12 September. This consisted of a visual index for each plant represented in a scale of 1 to 5:

1.- Total leaf-loss
2.- Leaf-loss and 25% or more leaves with strong symptoms
3.- Leaf-loss or 25-50% leaves showing strong symptoms
4.- Leaf-loss or less than 25% leaves showing strong symptoms
5.- No symptoms

Each plant was evaluated and an arithmetic mean was calculated:

- Plant index =∑(nº plants*index)/total plants.

Seed cotton yield was studied as the sum of the first and second collection in Kg/ha.

<u>Chemicals</u>. The standard phenolic and terpenic compounds (catechin, gallic acid, gossypol and Folin Ciocalteu's phenol reagent) were purchased from Sigma (St. Louis, MO, USA). Brotomax was supplied by Agrometodos, S.A (Madrid, Spain).

<u>Quantification of Phenolic Compounds and Gossypol (and its Derivates)</u>. Control plants and plants treated with Brotomax were collected and each of the four samples was divided into three approximately equal lots. The plant material was ground and shaken with dimethylsulphoxide (DMSO) (100 mg fresh weight/ml) for 2 h for extraction. Extracts were filtered through a 0.45 µm nylon membrane before analysis by spectrophotometry, using a UNICAM UV/VIS spectrometer UV2 (Unicam Limited, Cambridge, UK), to estimate total phenols, which were expressed as gallic acid equivalents (Folin Ciocalteu Method). Gossypol and its derivates was quantified by HPLC with a Hewlett-Packard liquid chromatograph (model HP 1050) (Hewlett-Packard Co., USA) fitted with a diode array detector (range scanned = 220 - 500 nm) to detected by spectra and quantify at 254 nm. In the HPLC analysis the stationary phase was a Sherisorb ODS-2 column (250 mm x 4 mm i.d.) with a particle size of 5 µm at 35ºC. As solvent we used: acetonitrile (A)/water (B), 25 to 95 % of A in 50 min. The eluent flow was 1 ml min^{-1}. Absorbance changes were recorded in the UV-V diode array detector at 254 nm. The amounts of the compounds were determined from the area given by the integrator using the response factor of the corresponding standards.

<u>Antifungal Activity of Cotton Extracts</u>. Isolates of *Verticillium dahliae* were supplied by the CCT from Spain, and cultured on potato dextrose agar (PDA) at 25ºC to serve as inoculums. The antifungal activity of cotton extracts from control and Brotomax-treated plants was determined by *in vitro* assay with the fungi as described in a previous paper (Del Río et al., 1998).

<u>Light and Electron Microscopy</u>. The microscopic examinations and the conditions and procedures used to process the stem were as reported in previous studies (Ortuño et al., 1990), which also described how semithin and ultrathin sections for light and electronic microscopy were obtained. A Photomicroscope II (Carl Zeiss, Oberkochem, Germany) was used for examination under the light microscope. For ultramicroscopic examination, the cotton sections were cut (Ultracut Reicher Jung), stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 electron microscope with an acceleration of 60 KV.

Results and Discussion

Microscopic Study of Vascular Occlusion

The general pattern of symptoms of Verticillim wilt in cotton is a total or partial loss of turgor, originating as flaccidity of the lowest leaf, or a terminal leaflet in a compound leaf developing towards the stem and spreading acropetally until the whole plant is affected, severe cases resulting in death. The initial flaccidity is followed in rapid sequence by chlorosis and necrosis. This partial loss of turgor is due to development of a vascular occlusion. Photo 2 shows a cross-section of an infected root. An obstruction and discoloration of the xylem can be seen. The vessel obstruction is due to the formation of tyloses (Photo 3), which are formed by expansion of xylem parenchyma cells (Del Río et al., 2001). These tyloses are formed in the xylem wall and grow to invade the vessel lumen. These structures were present in the roots and stem of the different cotton varieties assayed.

Another reason for the decreased water flow in the xylem is due to the development of gels and gums. So, Photo 4 shows the presence of polymers in the xylem lumen. In different paper related with *Fusarium,* Beckman and Talboys (1981) described the role of gels and gums principally as spore-trapping mechanisms that delay or prevent fungal spread.

The vascular occlusion is interpreted by some authors as a resistance mechanism, isolating the pathogens and restricting its spread (Beckman and Talboys, 1981). Others think that such mechanisms of vessel plugging are a feature of pathogenicity rather than resistance, and that the fungal pectolytic enzymes are involved in their development (Pegg 1981).

The development of these structures is due to the presence of mycelia fungi within the xylem, as can be observed in Photo 5. So, Verticillium and other fungi live inside the xylem vessel and colonize the plant by mycelia growth or by conidial production (Photo 6), traveling with the flow of water due to transpiration and germination in the stem xylem. This fungus invade different xylem through the pits from one vessel to another (Photo 7)

Increase of Biochemical Resistance of Cotton Against Verticillium by Brotomax

Cotton plants treated with Brotomax increase their total content of phenolic compounds and gossypol and its derivatives) in relationship to control plants. Figure 1 show the content in roots of Crema and Corona cotton varieties (Figure 1A corresponding to phenolic and Figure 1B to gossypol compounds). Similar increase was observed in different organs. The capacity of cotton extracts to inhibit Verticillium growth is shown in Figure 2. The presence of high concentration of phenolic and terpenic compounds in cotton plants treatment with Brotomax determine that the extract of this plants have high antifungal capacity or resistance against *Verticillium dahliae*. This increase of antifungal capacity was higher in stem and roots of cotton plants treated.

At the present time, there is a major underlying assumption that resistance is based on the exclusion or expulsion of pathogen from the host or its restriction within the vascular system. The implication of different compounds (phenolic and terpenic) in biochemical resistance has been described and there are many examples of increases in polyphenols in cotton following *V. dahliae* infection. The major phenolics are thought to be tannins (Bell, 1973; Mace et al., 1978), and Bell (1973) claimed that only a few specific tannin-producing xylem parenchyma cells exist in healthy cotton, whereas under infection stress most cells have this propensity. Also, the origin of vascular browning in cotton is thought to be condensation of oxidation products of catechin. Phenol concentration has been correlated with resistance of cotton species (Bhaskaran and Muthusamy, 1974). Pegg (1981) listed the roles of phenolic compounds in host physiology as: uncouples of respiration from oxidative phosphorylation, enzyme inhibitors, host growth inhibitors, antifungal compounds and enzyme stimulants. In relationship to terpenic compounds, gossypol is the principal terpenoid present in cotton plants and cotton seed oil. 6-methylgossypol ether and the 6,6'-dimethyl ether are major constituents of the root epidermis and the lysogenous pigment gland of the phloem (Mace et al., 1978; Bell et al., 1978). The major derivatives of gossypol, acting as phytoalexins, are hemigossypol and hemigossypol-6-methy ester, especially in resistant cultivars (Bell et al., 1975).

This increase in the antifungal activity of cotton extracts in plants treated with Brotomax affects vessel occlusion. In Photo 8 the effect of treatment of Brotomax on the vascular browning of roots and stem of the Crema variety can be observed. A reduction of vascular discoloration of 30% in roots and 50% in stem was observed for this verity. Similar effect was observed in the other cultivars assayed.

<u>Influence of Brotomax Treatment on the Yield and Visual Tolerance of Six Cotton Varieties Assayed</u>

The results of the effect of Brotomax on the yield and the tolerance are show in Table 2. As regards yield, cotton plants treated with Brotomax showed a mean increase of 773.04 $Kg.ha^{-1}$ (18.65%), although this data depended on the variety. For example, the increase for Carmen was 337.37 $Kg.ha^{-1}$ (8.83%) and for Crema 1078.11 $Kg.ha^{-1}$ (28.28%) (Table 2 and Figure3).

Tolerance was also observed to depend on variety (Table 2 and Figure 4), with a mean increase of 0.24 (7.18%), the lowest value being for Carmen 0 (0%) and the highest for La Chata 0.50 (23.07%) (Table 2). These results show that varieties susceptible to disease have a higher increase in visual tolerance, although increase yield due to treatment with Brotomax does not depend on the genetic tolerance of the variety.

The increase in yield and tolerance to Verticillium was significant at 0.01%, with a value of correlation of 0.56, which suggest that the tolerance visual is a good index of the production for all the varieties assayed. The differences between varieties and the repetitions was also significant but not between the variety and treatment. These results show that Brotomax can increase cotton yield and tolerance against Verticillium in the different varieties assayed (Table 3). Similar results were obtained by Gutiérrez et al. (2000).

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Table 1. Characteristic of tolerance and productivity of different cultivars assayed.

Cultivars	Tolerance to Verticillium	Productivity
Carmen	High	Médium-high
Delta Opalo (Delta Opal)	High	High
Corona (DP-20)	Médium	Médium-high
Crema (Stoneville KC-311)	Médium-high	Médium-high
La Chata (Cocker)	Low	Low
Conchita	Médium	Medium

Table 2. Effect of the treatment with Brotomax in yield (**Kg/ha**) and tolerante to *Verticillium* (Plant index).

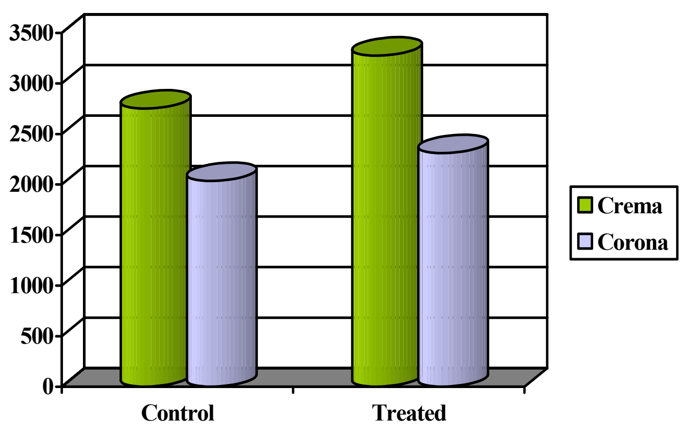
	Seed cotton yield		Plant index	
Cultivars	**Differences between treated and control**	**Increment (%)**	**Differences between treated and control**	**Increment (%)**
Carmen	377.37	8.83	0.00	0.00
Delta Opalo	997.05	19.57	0.19	5.13
Corona	487.93	10.35	0.32	10.44
Crema	1078.11	28.28	0.20	5.89
La Chata	1041.76	34.89	0.50	23.07
Conchita	656.00	16.43	0.25	6.25

Table 3. Media square of the variance analysis of the yield (Kg/ha) and tolerane to *Verticillium* (Plant index).

Source of variation	DS	Seed cotton yield ($Kg.ha^{-1}$)	Tolerance plant (Plant index)
Block (B)	2	658037.54*	2.22**
Cultivar (C)	5	2929167.00**	2.17**
B x C	10	211345.52	0.16
Treatment (T)	1	5378249.20**	0.53**
T x V	5	140098.82	0.04
Error	12	149238.22	0.03
CV (%)		8.52	5.28

* Significant to 5%
** Significant to 1%
CV: Coefficient of variation

A)



B)

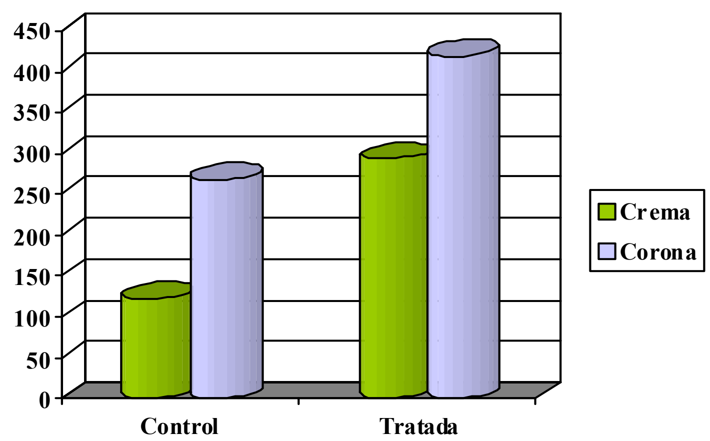


Figure 1. Effect of Brotomax on the phenolic total content (A) and gossypol and its derivates (B) in cotton roots of Crema and Corona recollected in August . Concentration expressed in mg/ 100g fresh weight.

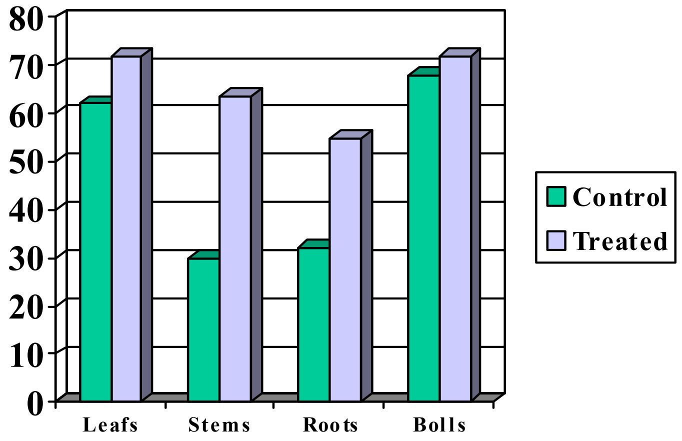


Figure 2. Inhibition of Verticillium growth (%) by cotton extracts of different organs of cotton plant (Crema) treated with Brotomax and recollected in August.

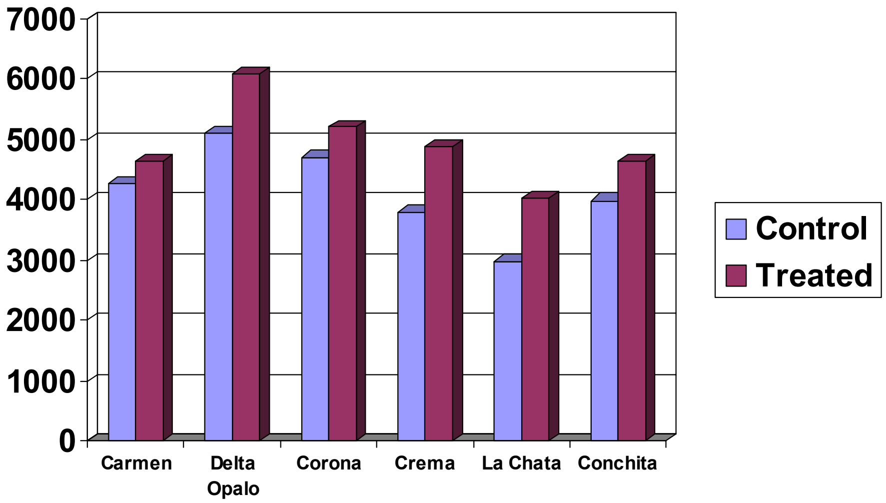


Figure 3. Effect of Brotomax on seed cotton yield (Kg/ha) of different varieties assayed.

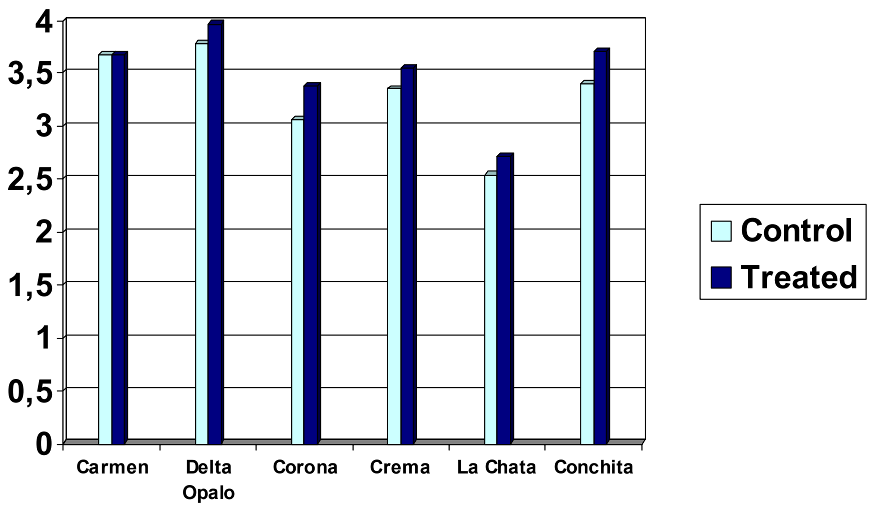


Figure 4. Effect of Brotomax on tolerance to Verticillium of different varieties assayed.

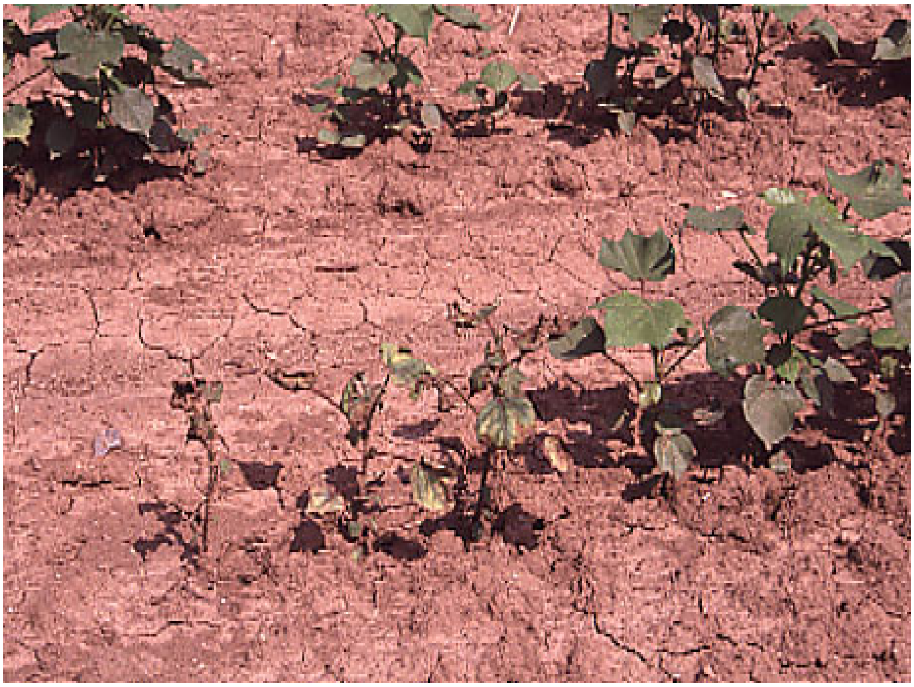


Photo 1. Symptoms early of Verticillium wilt in cotton plants (Photo realized by Jesús Rossi y Eva Braojos).

Photo 2. Cross-section of infected cotton root (cv. Crema). The discoloration of the xylem can be seen.

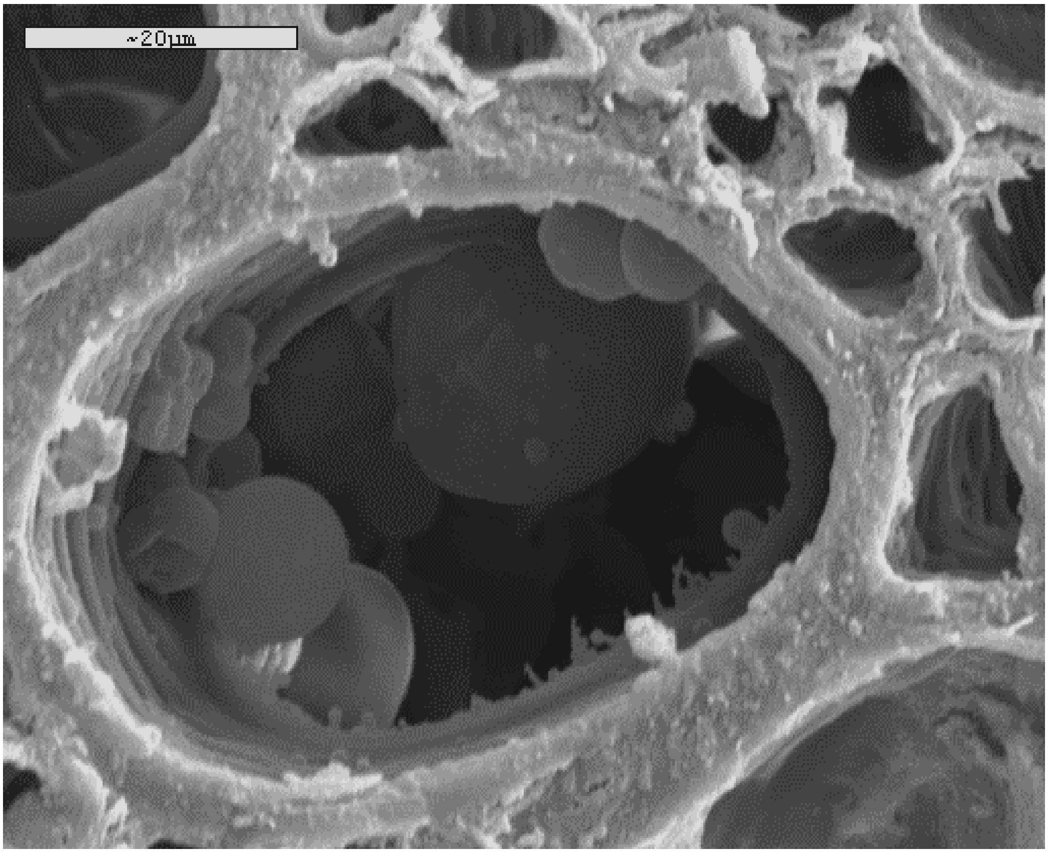


Photo 3. Scanning electron microscopy (SEM) of a cross-section of cotton root (cv. Crema). The presence of tyloses in the xylem lumen can be observed.

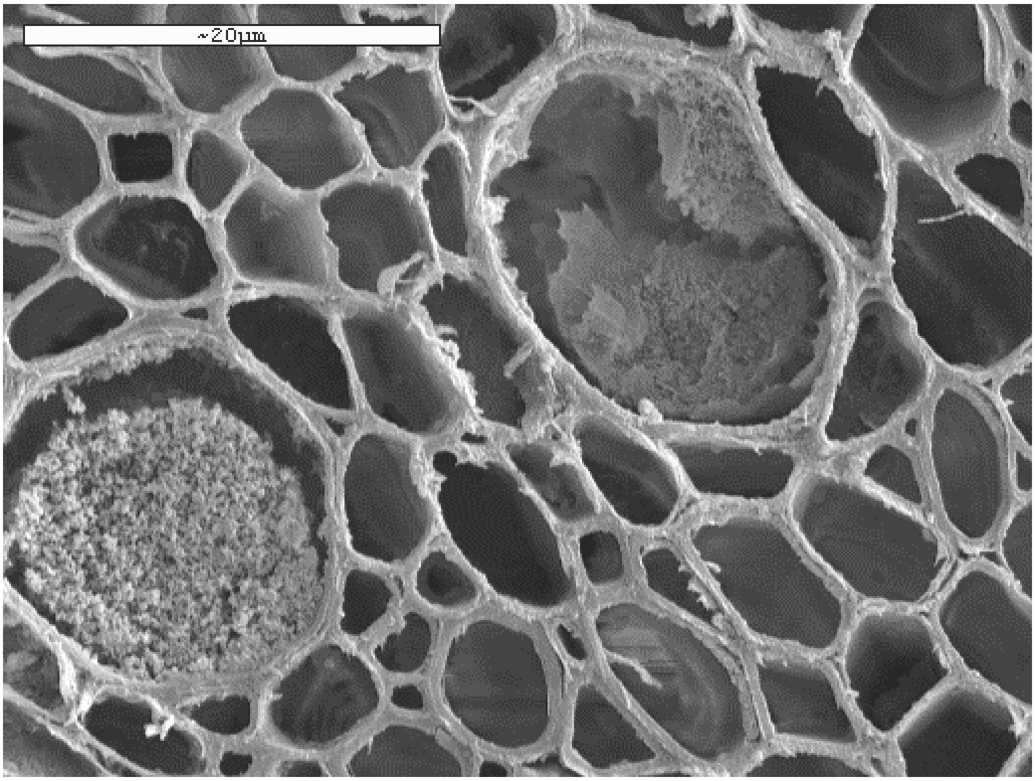


Photo 4. Scanning electron microscopy (SEM) of a cross-section of cotton root (cv. Crema). The presence gum into xylem (left) or tyloses in the xylem (right) can be observed.

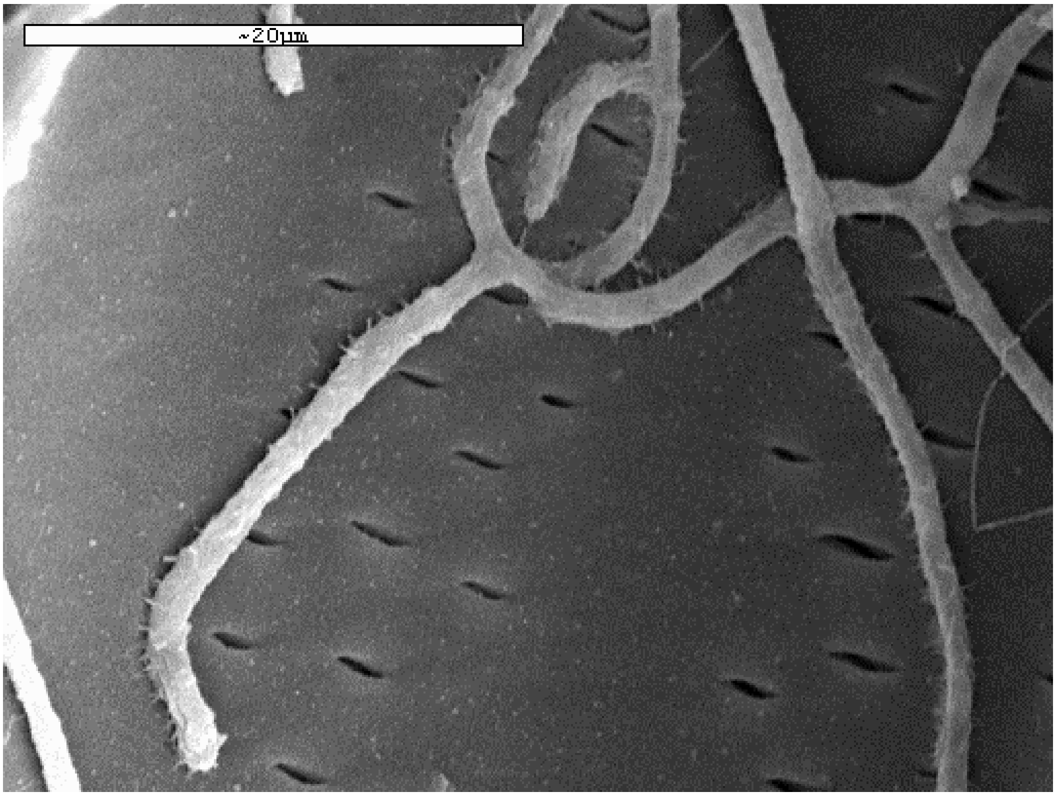


Photo 5. Scanning electron microscopy (SEM) of a longitudinal-section of cotton root (cv. Crema). The presence of mycelia fungi within the xylem can be observed.

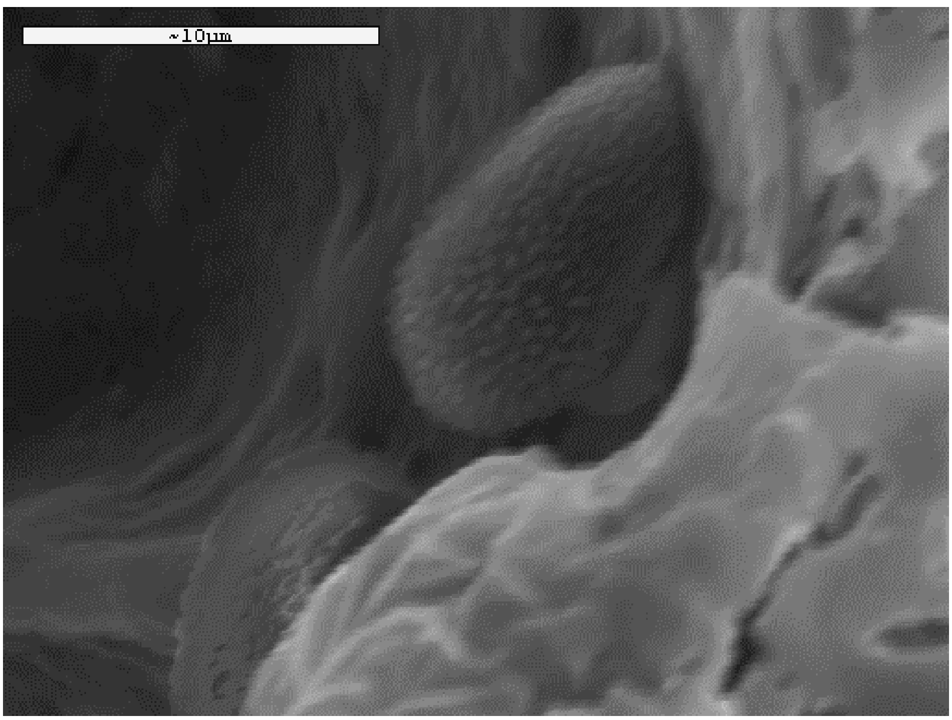


Photo 6. Scanning electron microscopy (SEM) of a longitudinal-section of cotton root (cv. Crema). The presence of Verticillium conidia in the xylem wall can be observed.

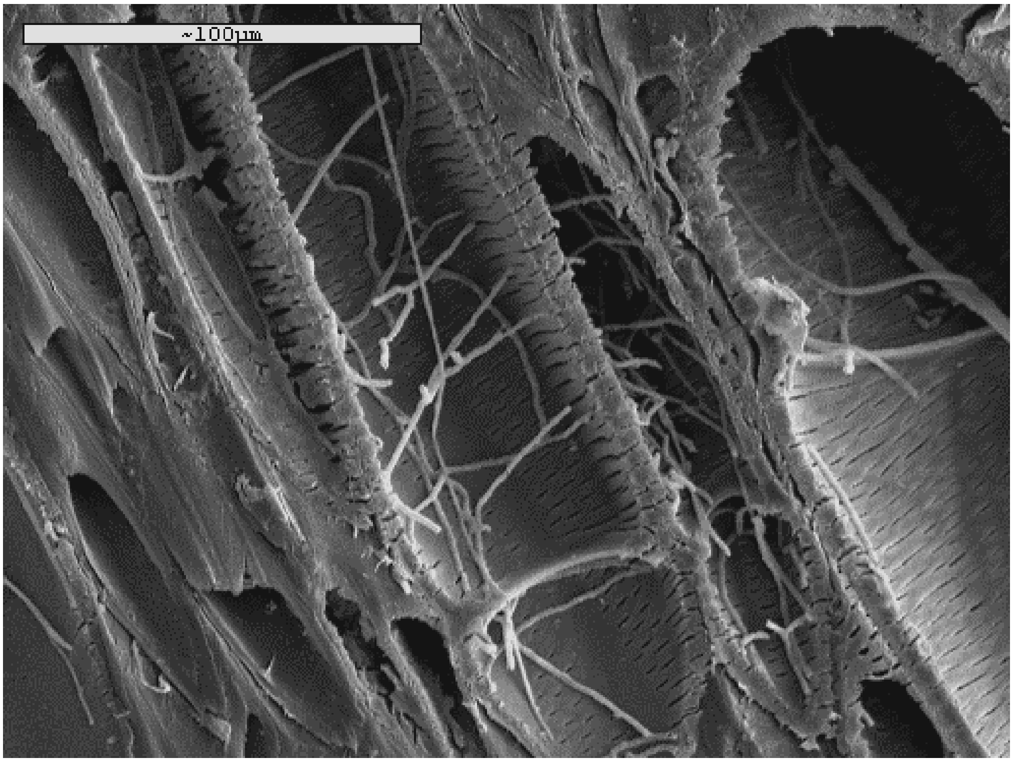


Photo 7. Scanning electron microscopy (SEM) of a longitudinal-section of cotton root (cv. Crema). The presence of mycelia fungi within the xylem can be observed. This fungus invade different xylem through the pits from one vessel to another.

Photo 8. Effect of Borotomax treatment on the discoloration of the xylem in stem of cotton plants (cv Crema). A reduction of vascular discoloration of 50% was observed.