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Effect of Antagonists and Plant Extracts in the Control of Protea Wilt (*F. oxysporum*)

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Abstract

In laboratory experiments were evaluated TRICHOD®, FOLIGUARD®, *Trichoderma* sp, and watery extracts of pine, marigold and eucalyptus, respectively, against *F. oxysporum.* There were no significant differences between TRICHOD® and FOLIGUARD®. The extract of marigold of 120 g/L was the best result in reducing the area of the colony of *F. oxysporum*. In field pots experiment were evaluated the extract of marigold (120 g/L), potassium phosphite (400 g/L), TRICHOD and benomyl (1.5 g/L) to control *F. oxysporum*. Treatments were applied to the soil, 15 days after inoculation with *F. oxysporum*. The best obtained heights were of 32 cm with extract of marigold 120 g/L, followed by potassium phosphite with 31 cm, and the control reached 26 cm of height. The treatment with an extract of marigold 120 g/L, obtained the best dry weight average of 6.5 g, statistically different from the control. The extract of marigold presented the best efficiency against the disease, because it reduced the length of the vascular symptom in 88.5%; followed by TRICHOD®, benomyl and potassium phosphite that showed reductions of 86, 85 and 84 percent, respectively.

Keywords

Marigold Extract, Plant Disease Control, *Trichoderma*

1. Introduction

The diversification in planting ornamental species has been a strategy used by the producers, as an alternative to extending markets, to improve competitiveness and to increase profitability. A step in this respect has been the production and commercialization of new flowers and foliages, as the proteas of the genus *Leucadendron.*

The cultivation of proteas is relatively new in the market of ornamental plants for the production of flowers,

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dried flowers and flower pots [1]. There is great diversity of proteas and the genus *Leucadendron* has stand out in international trade, mainly in the European community, as cut and processed as dried flower blossom. The main consumers of proteas are Japan, Europe and the United States. This market has an estimated value of 455 million euros. The total number of stems exported around the world is 100 million units and about half of total world exports are of the genus *Leucadendron* [2].

Proteas production has been affected by the incidence of diseases caused by three pathogens of great importance that inhabit the soil: *Phytophthora cinnamomi*, *Rhizoctonia* spp. and *Fusarium* spp. [3]-[5]. In the genus *Fusarium* three species are agents of *Leucadendron* wilt: *F. oxysporum*, *F. solani* and *F. moniliforme*. *F. oxysporum* was first detected in the northwestern region of South Africa and Zimbabwe [6]. It has subsequently been reported in different parts of the world where proteas are cultivated and it was reported the incidence of *F. solani* and *F. equiseti* attacking proteas of the genus *Leucandendron* especially in the Madeira Island in Portugal [3]. It has also been found in the Canary Islands [7] and in Australia [8]. In Australia, was found *F. oxysporum* in roots, neck and vascular tissue of plants with wilt symptoms [9]. Also, has been detected in seedlings and commercial plantations, causing losses greater than 60% [8]-[10].

In Colombia, *F. oxysporum* is the causal agent of *Leucadendron* wilt [1]. Symptoms have been observed in young and adult plants of the protea cultivars Petra and Gold Strike. Necrosis was observed at the beginning in young leaves and side shoots that die after infection, generating a reduction in the number of flowers produced per plant [6]-[8]. Vascular tissues acquire a dark brown color which extends from the root to the stem of the plant and affects both the xylem and phloem. There is a turgor loss in the apical part of the shoot, yellowing occurs from the lower leaves towards the top of the branch. Subsequently, the branches weaken and necrosis appears in leaves and the stem [1].

In general, the diseases caused by *F. oxysporum* are difficult to control. Resistant varieties, planting certified seeds, and healthy seedlings are the best practices to control wilt caused by *Fusarium* species. Also antagonist biocontrol agents, such as *Trichoderma*, offer possibilities to control, primarily because it inhibits the growth and development of the disease [11]. The genus *Trichoderma* has fast reproduction; it is able to survive and to modify the rhizosphere and it is effective in promoting plant growth [12]. The incidence of proteas wilt was reduced to 20% by using a native strain of *T. harzianum* in concentrations of 10^8 and 10^9 conidias per mL [7]. *Tricoderma* has been commercially produced to be used in controlling several plant pathogens such as: *Pythium* spp, *Rhizoctonia* spp, *Sclerotium* spp, and *Fusarium* spp [13].

A variety of *Trichoderma* species has been evaluated to control *F. oxysporum* in different crops. To control this pathogen in papaya plants (*Carica papaya* L.) a treatment of *Trichoderma* at a concentration of 10^6 conidia wasapplied to the soil artificially infested with *F. oxysporum*. Its antagonist effect has also been evaluated on tomato plants to wilt caused by *F. oxysporum* [14].Tomato seeds coated with *T. harzianum* were planted in natural soil artificially infested with *F. oxysporum* [15]. The seed treatment caused no reduction in the disease, whereas the soil treatment was able to reduce 92% of the infection. In Cuba *T. harzianum* applied 20 g/plant in soils with occurrence of Panama disease, provided superior control to 95% in the varieties tested [16].

In searching for new alternatives to organic plant disease management, the use of plant metabolites has been considered feasible [17]. Botanical pesticides are chemical compounds that occur naturally in plants. They have been used to reduce the growth of certain fungi under laboratory conditions. Extracts of eucalyptus and other plants in different concentrations reduced the growth of *F. solani*. The marigold (*Calendula officinalis*) is cultivated in different countries, and some studies have been developed using this plant extractto control some species of nematodes that attack citrus, but its greatest use has been in human dermatology and food industry, due to its antimicrobial properties [18] [21]. Essential oils are extracted from roots, seeds, leaves and flowers of marigold, which are rich in sesquiterpenes, phenols and saponins that are responsible for the antifungal activity [22]. Sesquiterpenes activity was evaluated *in vitro* for control *C. acutatum*, *C. fragariae*, *C. gloesporoides*, *F. oxysporum*, *Botrytis cinerea*, and *Phomopsis* sp [23].

In Colombia, the growers apply some chemical fungicides, such as benomyl to wilt control. This product reduced by 50% the incidence of wilt caused by *F. oxysporum* on susceptible tomato plants [24]. Similar results were obtained in tomato [25] and susceptible native bean varieties planted in soil naturally infested with *Fusarium* spp and *Rhizoctonia solani* [26]. Also it reduced the Panama disease (*F. oxysporum. f. sp. cubense*) in banana by 70% with a drench application one week after transplantation was made [27]. According to Obreque [7] benomyl has been widely used in the preventive control of wilt of proteas in South Africa.

The antimicrobial properties of the phosphites were found in studies aimed at controlling diseases caused by

oomycetes [28] [29]. Also, Mogollón and Castaño [30], showed the effect of potassium phosphite as resistance inductor, when it was applied to the soil to control sigatoka disease caused by *M. fijiensis* and *M. musicola* in plantains. And Walters *et al*., [31] demonstrated the effectiveness of potassium phosphiteto control different diseases, including those caused by *Phytophthora infestans*, *Fusarium oxysporum* and *Rhizoctonia solani*. It was found that the foliar application of potassium phosphite reduced by 40% the severity of wheat wilt caused by *F. culmorum* [32].

The main objective of this research was to determine the effect of *Trichoderma* spp, potassium phosphite, benomyl and plant extracts in the control of *F. oxysporum* in *Leucadendron* proteas, in laboratory and field pot conditions.

2. Materials and Methods

2.1. Laboratory Experiments

F. oxysporum was isolated from diseased *Leucadendron* proteas collected in established crops, in a farm located in Subachoque, Department of Cundinamarca, Colombia, at 2600 altitude, and temperature between 15˚C and 19˚C. Wilty Petra variety plants were chosen with external and internal symptoms. The fungus was isolated in acidified Potato Dextrose Agar (PDA + A) with 2500 µL of 50% lactic acid per 1000 mL of medium. Stem samples with visible wilt symptoms were previously washed with tap water to remove soil residues. Of each stem, square 5 mm pieces were cut, and immersed 1 min in 70% ethanol, two minutes in sodium hypochlorite 1% and washed in sterile distilled water for 30 s [33]. In a laminar flow cabinet, four pieces were planted on each Petri dish containing PDA + A using a sterilized forceps. The isolated samples were incubated at 20˚C for eight days. On slides, stained with lactophenol cotton blue, were examined at light optical microscope (Nikon eclipse E 100) at 40× and 100× to check the morphological characteristics of the pathogen, by comparison with diagrammatic keys [34] [35].

Data from each of the two laboratory experiments, were processed with the SAS statistical program, through which the fulfillment of assumptions prior to the execution of the analysis of variance was tested. In cases where the F-test analysis of variance was significant, the honest Tukey multiple comparison (Tukey HSD) was used to determine the differences within treatments.

Experiment 1. The TRICHOD® (Orius Biotecnologia) and FOLIGUARD® (Live Systems Technology) commercial biological products with the active ingredient *Trichoderma harzianum* and an isolate of *Trichoderma* sp uncharacterized, were evaluated against *F. oxysporum* using spore suspensions at a concentration of 1×10^8 spores/mL. A completely randomized design was followed, with 15 replications, where each treatment corresponded to a biological product. Two discs of 8 mm diameter, one of the pathogens and one of the antagonists, were transferred to a Petri dish with PDA + A. These discs were located one in front of the other at a distance of 3 cm, and were incubated at 20˚C. The pathogen area growth in each treatment, and the control without treatment was measured three times at week for a period of three weeks.

Experiment 2. In order to set the action of plant extracts of pine, eucalyptus and marigold on the development of colonies of *F. oxysporum*, the next procedure was followed: Samples of these plant species were collected in the campus of UDCA, washed with water, immersed in sodium hypochlorite 1% for two minutes, and finally rinsed with sterile distilled water. Three subsamples of 60 g, 120 g and 180 g, for each one of pine, eucalyptus and marigold samples, respectively, were evaluated. Each subsample was boiled in a liter of sterile distilled water and subsequently filtered through filter paper, adjusted to 1000 ml of water to prepare a culture medium PDA + A + Extract (PDA + A + E).

From the pure culture of *F. oxysporum* obtained in experiment 1, 15 discs of 8 mm in diameter were extracted and planted in individual petri dishes containing the PDA + A + E culture medium, and incubated at 20˚C. The colony area was measured three times a week for a period of three weeks. It was followed a completely randomized design with 15 replications for each extract and concentration.

2.2. Field Pots Experiment

Leucadendron proteas plants, cultivar “Safari Sunset”, one month old at the time of transplantation, with no internal and external disease symptoms were planted in 5 kg pots, filled with unsterilized soil obtained in the experimental field of UDCA. Once planted they were located according to the experimental design in rows of 10

pot plants per each of the five treatments, spaced 0.30 m apart.

Then, a randomized complete block design was followed with five treatments and five replications. The treatments were *T. harzianum*, marigold plant extract 120 g/L, potassium phosphite, benomyl 50 WP, and the absolute control. The treatments had randomization within the blocks and in the experimental area. Each experimental unit consisted of 10 plants for a total of 250 plants. For data analysis, the Minitab 16 statistical software was used.

To isolate *F. oxyporum* it was followed the procedure previously described. Ten days after planting, the seedlings were inoculated with a suspension of 10^6 macroconidia/mL of *F. oxysporum* [Salazar *et al*., 2010]. One mL of the suspension was injected into the root collar, using a plastic syringe. Fifteen days after the inoculation the following treatments were applied: 1) 5 mL per pot around the plant of a suspension of TRICHOD® at a concentration of 2×10^{10} conidia/mL of *T. harzianum*; 2) This treatment consisted of the application of 10 mL of marigold extract, around the neck of the plant. The marigold extract was prepared with 120 g of marigold leaves per liter of water, because the best results were obtained in the laboratory study; 3) For the treatment with the fungicide the product benomyl 50 WP was used in doses of 1.5 g per liter of water. Two mL of the solution were used to drench the soil around the stem in each plant; 4) To evaluate the potassium phosphitethe commercial product Agrifos 400 SL, was used. The drench had a concentration of 7.5 mL of the commercial product per liter of water. Each plant was drenched with 5 mL of this solution; 5) The control did not receive chemical nor biological treatments.

For six months, every 15 days data were taken in five randomly selected plants in each treatment and replications. a) The height of the plant was measured between the soil surface and the apex of the last leaf of the plant with a ruler; b) The number of side shoots was quantified; c) At the end of six months, no external symptoms of the disease manifested and for this reason destructive sampling was performed in order to observe wilt symptoms in the vascular bundles of the plant. Dry matter accumulation was determined in the roots of the plants selected at random, in the destructive sampling. Fresh weight was taken and then placed in an oven for 48 hours at 60˚C, to obtain dry weight. Five plants per treatment were evaluated, totaling 25 plants. For data analysis the Minitab 16 statistical software was used and the data were subjected to analysis of variance, Tukey test and the examination of contrasts.

3. Results and Discussion

3.1. Experiments at Plant Pathology Laboratory

Significant differences were found ($p < 0.01$) among three treatments: TRICHOD®, FOLIGUARD® and *Trichoderma* sp, and the control. In general, they reduced the growth of the *F. oxysporum* colony. Duncan test indicates that TRICHOD® reduced significantly the colony area of *F. oxysporum*, followed by FOLIGUARD® and *Trichoderma* sp (**Figure 1**).

The results indicate that *Trichoderma* inhibits colony growth of *F. oxysporum* under laboratory conditions. This antagonistic effect has already been described by other researchers to explain the potential of the genus *Trichoderma* for the management of this important plant pathogen. Cifuentes [36] found that *T. harzianum* was

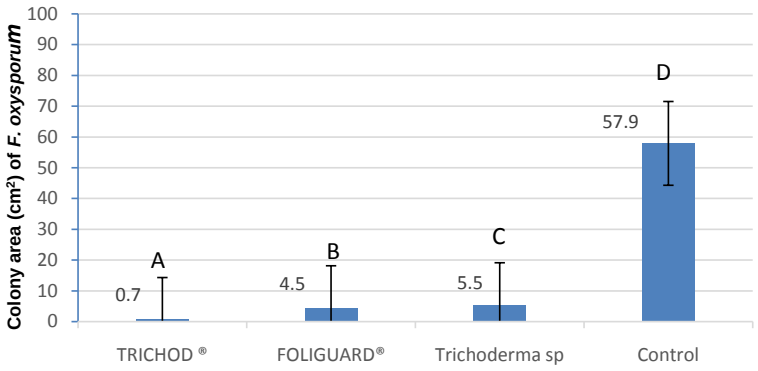

Figure 1. Effect of TRICHOD®, FOLIGUARD® and *Trichoderma* sp on the colony area of *F. oxysporum*. Means of 15 replications. Means with the same letter are not significantly different by the Duncan test.

highly effective in controlling *Fusarium solani* in tomato. Also, when the chemical control and the use of antagonists against *F. graminearum* were compared, it was determined that copper oxychloride inhibited growth of the pathogen, whereas *Trichoderma* reduced 64% the colony growth and was effective in reducing the severity of the disease in wheat crops [37]. Meanwhile, it was established [16] that *T. harzianum* obtained 95% control in plantations of banana affected by Panama disease.

According to Stefanova *et al*., [38] and Selosse *et al*., [39], the activity and biocontrol efficacy of *Trichoderma* is based on the property to act directly as hyperparasite, and indirectly by means of the action of antifungal metabolites and hydrolytic enzymes that cause cellular changes in the pathogen, and may cause degradation of the cell wall of *F. oxysporum*. These characteristics of *Trichoderma* will increase their competitiveness and effectiveness in controlling other pathogens that inhabit the soil.

The tested plant extracts showed significant differences ($p < 0.01$) among treatments (**Figure 2**) and reduced the colony area of *F. oxysporum* and best results were found with marigold extract. The three concentrations evaluated were significantly different from other treatments and the control, but no statistical differences were found between the three concentrations of marigold. Treatment corresponding to 120 g, had the lowest growth area of *F. oxysporum*. No differences were found between pine and eucalyptus extracts and the control.

There is a possibility of using plant extracts to control plant pathogens. In coffee plants promising results have been obtained in the management of diseases because of their mechanism of direct action on pathogens, and their potential for inducing systemic resistance [40]-[43]. The antimicrobial action of marigold extract is linked to the production of secondary metabolites content in vegetables such as flavonoids, salicylic acid, sesquiterpenes and phenols [23]. For example, phenols, are toxic against *Fusarium* species [41]. Eucalyptus extract has been found to reduce the growth of mycelium of *Colletotrichum*, but no effects on *Fusarium* are known [42].

The results explore the possibility of new organic technologies that allow a sustainable management of plant health problems, which counteract the harmful effects of the use of synthetic chemical fungicides. New technologies are a common benefit to produce proteas and other crops where *Fusarium* attacks generate large losses. Moreover, the results are a significant contribution to knowledge and use of biodiversity, because working with native and introduced plants, and domesticated microorganisms in Colombia generate knowledge to expand their potential uses.

3.2. Field Pots Experiment

Plant height. The data were transformed, and significant differences ($p < 0.001$) among treatments and blocks were found. The heights were 31 cm and 32 cm between plants treated with potassium phosphite and plants treated with marigold extract, respectively. These results were statistically different to the control (**Figure 3**). Theapplication of benomyl, could have an initial effect on the incidence of the disease, but was not persistent

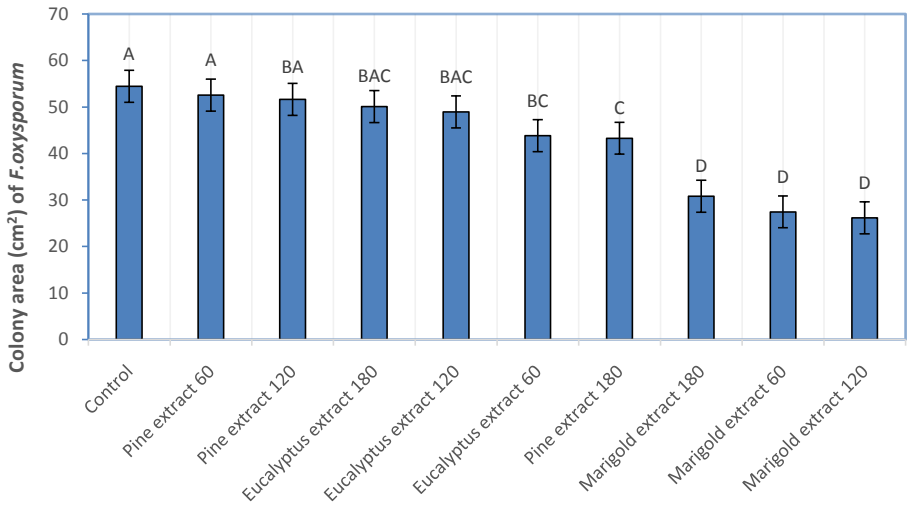

Figure 2. Effect of pine, eucalyptus and marigold extracts on the colony area of *F. oxysporum*. Means of 15 replications. Means with the same letter are not significantly different by the Duncan test.

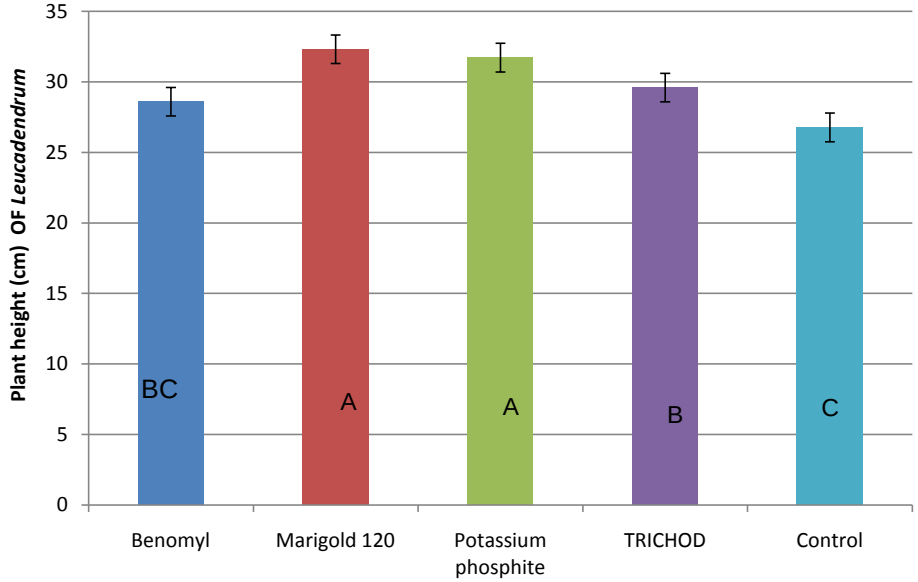

Figure 3. Effect of benomyl, marigold 120, potassium phosphite and TRICHOD on plant height of *Leucadendrum*. Means of 5 replications. Means with the same letter are not significantly different by the Tukey test.

enough to allow normal plant development. The control presented a marked delay in growth with 26 cm and development compared to treated plants.

Number of side shoots. There were no significant differences ($p \leq 0.05$) among the treatments: TRICHOD, potassium phosphite, marigold extract and benomyl. However, there were differences between these treatments and the control. The greatest number of side shoots was 2.5 occurred with the TRICHOD® treatment (**Figure 4**). Avis *et al*., (2008) highlights the action of *Trichoderma* not only as a biocontrol agent, but also as a growth promoter. Different strains of *T. harzianum* and *T. viride* have shown increases in fresh and dry weights of lateral stems; also the increase in volume and dry weight of roots have been found in vegetables and other crops such as tobacco. In addition, two mechanisms of action have been identified with respect to the benefits of *Trichoderma* on plant growth. The first is an increase in nutrition through the solubilization and absorption of micro and macro nutrients, and the second is production-related growth promoters in plants, such as indol acetic acid [43]. Side effects of the antagonist found in this research are important because the productivity of cut flowers is measured by the number of lateral stems with flower. In plantations, it is possible to reach better performance with this treatment, even with some incidence of the disease. Marigold treatments, potassium phosphite and benomyl, were, 2.5, 2.3 and 2.4 side shoots per plant respectively, exhibiting difference versus the lowest average of 2.2 lateral shoots per plant. In addition, there is a confirmation of the effect of *F. oxysporum* on the development and growth of the plant when it does not receive any treatment.

3.3. Root Dry Weight

Treatment with marigold extract 120 g/L, had the best average of 6.5 g and it was statistically different from the control (**Figure 5**), but there was no difference between the marigold, potassium phosphite, benomyl and TRICHOD® treatments. When the dry matter accumulation in the root and the average plant height are compared, similar behaviors are observed. A larger and well-structured root may provide greater anchorage and nutrient uptake to the plant, allowing better growth and development (**Figure 5**). *F. oxysporum* causes damage to the root, affects the amount of functional roots and decreases the amount of water absorbed by these. Some vascular pathogens are related to the inhibition in the production of root hairs, which reduces the absorption [11].

3.4. Disease Incidence

The disease did not show any external symptoms after six months of performing the inoculation. However, when the destructive sampling was done, it was found 100% incidence in all treatments. In the destructive sam-

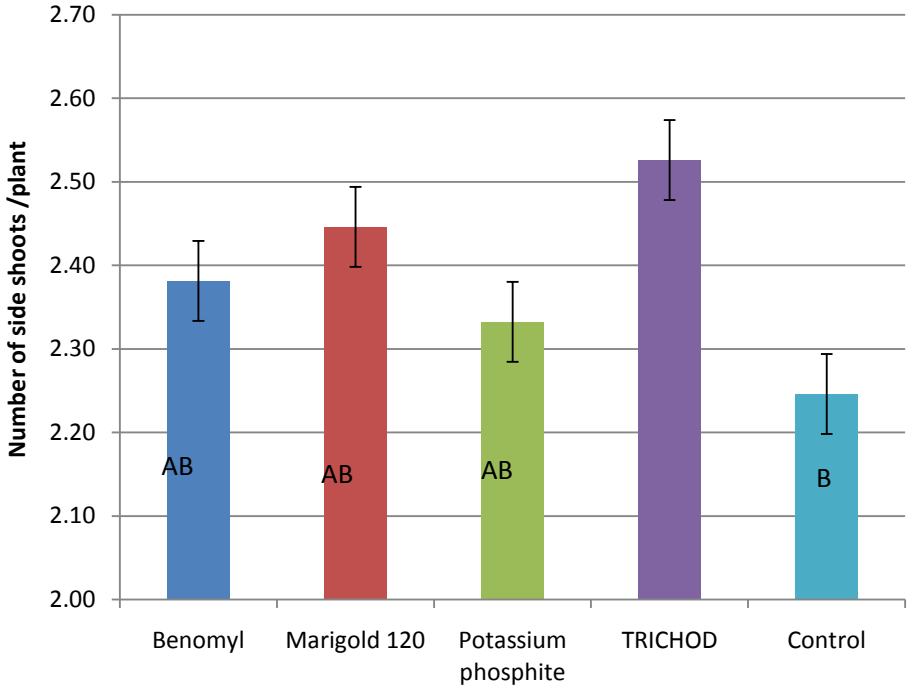

Figure 4. Effect of benomyl, marigold 120, potassium phosphite and TRICHOD on the number of side shoots of *Leucadendrum* plants. Means of 5 replications. Means with the same letter are not significantly different by the Tukey test.

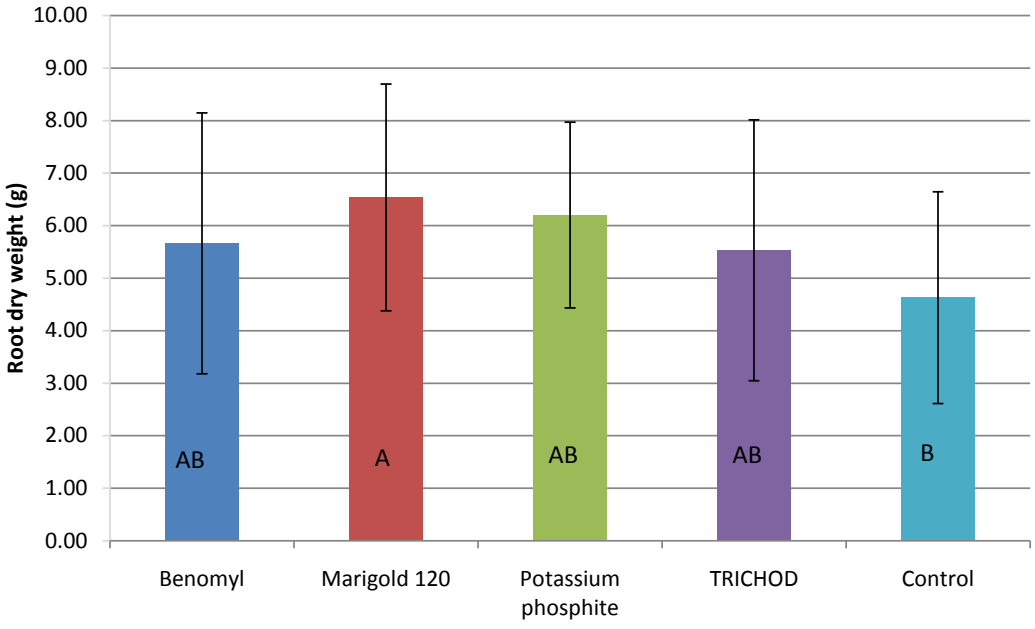

Figure 5. Effect of benomyl, marigold 120, potassium phosphite and TRICHOD on the root dry weight of *Leucadendrum* plants. Means of 5 replications. Means with the same letter are not significantly different by the Tukey test.

pling, it was evident the manifestation of symptoms in the vascular bundles, characterized by brown color around the xylem, extending from the base of the stem to the lateral side stems.

There were significant differences ($p \leq 0.001$) between treatments. The marigold extract presented the best efficacy against the disease, because reduced damage in 88.5%, followed by TRICHOD, benomyl and potassium

phosphite, which showed reductions in 86%, 85% and 84%, respectively. Although the treatment of potassium phosphite was not superior to marigold extract, it did show good plant height average (**Figure 3**), and it was able to reduce the development of disease. This result could be related to the induction of resistance mechanisms that allowed the plant to generate tolerance to the disease, and continue their growth and development. These results agree with the work of Monsalve *et al*., [44] who evaluated the effects of induced phytoalexins from phosphites in Oomycetes, *Rhizoctonia solani* and *Botrytis cinerea*.

Overall, marigold extract produced the best results in this investigation. Marigold has a number of compounds, such as sesquiterpenes, sesquiterpenoles, saponins and flavonoids that give certain anti-fungal properties. Martinez [22] mentions that flavonoids are aromatic compounds possessing anti-fungal activity against pathogens such as *Penicillium* sp and *Rhizopus* sp, amongst others. Phenolic compounds pervade pathogen cell membranes, resulting in leakage of cytoplasmic contents. According to Wedge *et al*. [23], sesquiterpenes are a family of more than 5000 compounds found primarily in members of the Compositae family. Sesquiterpenes have a broad spectrum of biological activity that plays an important role in the defense mechanisms of the plant. The same author found that sesquiterpene lactone had an inhibitory action against *Colletotrichum*, *Phomopsis*, *Botrytis*, but not against *F. oxysporum* isolated from *Leucadendron* sp.

Treatment with *T. harzianum* did not provide outstanding averages in the expression of growth traits, except the number of side shoots, which differed from the others; however, presented a significant reduction in lesion length, compared with the control. Possibly, the application of *T. harzianum* must be done before the infection, for greater efficiency, because, as demonstrated in the results obtained by Perez *et al*. [16], there was a complete control of *F. oxysporum* in banana plants treated with *T. harzianum*.

In contrast test was found that the length of the lesion was significantly higher in benomyl treatment versus marigold extract, indicating that the latter was better than the chemical treatment. Studies developed by Amini and Sidovich [25] indicate that better control of *F. oxysporumf.* sp. *lycopersici* was achieved with the application of pre-infection fungicide. This explains that the time of application affects the fungicide control responses of the disease. However, it is different with results obtained by Obreque [7], who obtained a 100% decrease in the *Leucandendron* disease when it was applied 30 days after inoculation. This result could be associated to the variability of *F. oxysporum* or the fungicide application method used.

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