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Field evaluation of biological control agents and calcium-based fertilizers to control *Plasmodiophora brassicae* in broccoli (*Brassica oleracea* var. italica)

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ABSTRACT

Broccoli plants are frequently attacked by the biotrophic pathogen *Plasmodiophora brassicae*, which leads to continuous significant economic losses during its cultivation. Finding environmentally friendly methods to control this aggressive pathogen is a priority. Here, broccoli plants were sown in a field that has been previously reported with a high incidence of the pathogen showing dramatic root damage and yield loss. Field evaluations were performed to evaluate lump weight, root weight, and lump diameter after soil application with *Trichoderma* spp., *Bacillus sp.*, humic acids, commercial plant defense elicitor, calcium carbonate ($CaCO_3$), and calcium nitrate ($Ca(NO_3)_2$). Treatments were applied individually and in combination during two continuous and independent production seasons (rainy and dry seasons) in the year 2018.In summary, the application of $CaCO_3$ increased the lump weight by 25% respective to its control, but only during the dry season. Moreover, the best treatment giving a consistent increase in lump weight (20% to 50% compared to its non-treated control depending on the season) was the combination of all treatments (biological and calcium-based fertilizers) when tested during both seasons. Taking all together, it was found some alternatives that increased the production of broccoli in fields infested with *Plasmodiophora brassicae*. These alternatives will be validated in the future in different soil types and agricultural environments.

Keywords: Broccoli, *Plasmodiophora brassicae, Trichoderma, Bacillus, Calcium.*

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INTRODUCTION

Broccoli is an important source of nutrients and minerals for the human diet. In 100 gr of broccoli, there is 56 mg of calcium, 22 mg of magnesium, 370 mg of potassium, 87 mg of phosphorus, 87 mg of vitamin C, and 69 µg of vitamin A (Aljaro, 2000). The demand for broccoli increases worldwide by 4% annually due to its essential health benefits. Specifically, it has been reported to have a beneficial effect against various types of cancer, such as lung, prostate, and breast, since its high content of antioxidant compounds like -carotene (1.9 mg -carotene per 100g of fresh broccoli) (Aljaro, 2000). The leading producers of broccoli are China with 39%, India with 36%, the United States of America (US) with 5%, Mexico with 2%, and others with 18%. Together they produce 24.2 million tons per year (Zilli, 2018). However, broccoli's leading exporters are the European Union with 52%, China with 17%, and Mexico with 12% (MCE, 2018). Ecuador participates with 2% of broccoli exports worldwide, reaching 6000 production areas (MCE, 2018). Thus, broccoli cultivation represents an essential aspect of the economy and agriculture in the Andean region of Ecuador.

From the farmer side, twenty percent of the production budget is destined to control pests and diseases. Broccoli's major diseases are: *Hyaloperonospora brassicae, Alternaria* *brassicae*, and *Plasmodiophora brassicae*. Plant diseases are challenging to control during the rainy season, leading to significant losses in production. The main strategy to manage plant diseases is based on chemical control (Haroand Maldonado, 2009).

P. brassicae is a disease that attacks Brassicaceae species. In Broccoli (*Brassica oleracea* var. italica), this disease affects the roots, causing deformation and weakness of the root system, which decreases the absorption of nutrients and can even cause mortality (Haro and Maldonado, 2009). This disease is considered the most dangerous in the broccoli monoculture system since it is difficult to detect it during the initial stages of the infection and later for its aggressiveness (Haro and Maldonado, 2009). According to Botero *et al.* (2019), *P. brassicae* decreases the yield between 10% and 30%. Because of the damage caused during broccoli production and enormous economic losses, it is crucial to define its control and management mechanisms.

P. brassicae is a protozoan with a complex life cycle that is not entirely understood since it consists of different zoosporic stages for the formation of plasmodia within host cells and the formation of resting spores (Schwelm, *et al.*, 2015). The haploid resting spore releases a zoospore that infects the hairs of the plant root and forms multinucleated plasmodia. These develop into several secondary zoospores, each with an individual nucleus that is released into the soil. A fusion of these secondary zoospores can occur occasionally. Zoospores invade the root cortex and develop secondary multinucleated plasmodia. This is where meiosis occurs in plasmodia before the formation of resting spores (Schwelm *et al.*,2015). The resting spores are extremely resistant to harsh environmental conditions and contaminate arable land for decades, making it difficult to eradicate the organism through any agrochemical or conventional soil treatment (Schwelm *et al.*,2015).

Several alternative measures have been proposed for the control and proper management of this disease, such as applying different sources and concentrations of calcium (Klasse, 1996), nitrogen (Ruaro *et al.*, 2009), and pH changes in the soil. It has been reported that the use of calcium carbonate ($CaCO_3$) and calcium nitrate ($Ca(NO_3)_2$) decreases the percentage of diseased plants by 20% and 10 %, respectively (Ruaro *et al.*, 2009). It has been previously shown that calcium has a beneficial growth effect on the crop in the presence of *P. brassicae* (Dixon, 2010). Besides, it has been shown that the use of calcium carbonate can increase the pH of the soil, which causes a decrease in root damage near the end of the production cycle (Haroand Maldonado, 2009). However, the use of these fertilizers can affect the progress of pathogens and the soil's condition, altering the pH of the soil, affecting the microbiological balance, and generating physiological changes for the plant (Agris, 2016).

Due to this, biological control alternatives have been proposed to manage this disease, leading to increased productivity with low environmental impact. Beneficial fungi, such as *Trichoderma* spp., have been used as an alternative due to their antagonistic effect, phosphate solubilizing activity, growth promotion, and ability to increase plant defense (Camargo and Ávila, 2013). The *Bacillus* species (bacteria) is another alternative, as it has been tested as a biological control against *P. brassicae* (Xing-Yu *et al.*, 2012). Plant defense enhancers or elicitors have been proposed, as it allows a plant to generate a metabolic response after the infection by a pathogen (Mogollónand Castaño, 2011; Venegas-Molina *et al.*, 2020). An elicitor stimulates the production and accumulation of phytoalexins that are toxic for a broad spectrum of phytopathogenic bacteria/fungi. It is also associated with the induction of defense genes that depend on the production of salicylic acid and jasmonic acid hormones (Riveros, 2009; Pieterse *et al.*,2012). Additionally, humic acids correspond to a mixture of organic aliphatic and aromatic acids, resulting from the degradation of organic matter that is not soluble in water when they are in acidic conditions. They are, however, soluble in water in alkaline conditions, which is why humic acids correspond to the proportion of humic substances that precipitate from aqueous solutions at a pH lower than 2 (Pettit, 2016). For this property, they act as a buffer in the soil, neutralizing the pH and generating an appropriate environment for developing the root system and beneficial micro biome (Pettit, 2016). None of these alternatives has been evaluated in broccoli combined with a specific concentration, making this study relevant.

The present investigation was carried out in 2018 in the Province of Cotopaxi, Ecuador. The aim of this study is to evaluate during two continuous harvest periods (rainy and dry season), the effect of the application of biological control agents and calcium-based fertilizers to increase broccoli yield in a field.

MATERIALS AND METHODS

Experimental Area

The study was carried out in the canton Latacunga of the Province of Cotopaxi at 0º48'10.51 "S and 78º36'58.63" W, with an elevation of 2932 meters above sea level (Finca AGROGANA, Piedra Colorada). The experiments were done in 1125 m^2 distributed in 25 m^2 plots of a broccoli field. Treatment was performed in an "old" broccoli field infested with *P. brassicae* (over 90% of infection). The plant material used was the cultivar Avenger, an important cultivar in the market due to its wide adaptation and constant yield.

Treatments and evaluated variables

Control treatment consisted of farm management where fertilizers and foliar chemicals were used to produce conventional broccoli. The treatments were based using conventional farm management plus the following active ingredients: calcium carbonate, calcium nitrate, *Trichoderma* spp. (concentration 1 x 10^9cfu per mL; T), *Bacillus* sp. (concentration 1 x 10^{11}cfu per mL; B), humic acids (a.i.80%; HA), and a mix of defense elicitors molecules (Quitosan, Potassium Phosphite, Folic Acid, and saccharin; E). These products were obtained from local commercial companies described in Table 1. All were tested alone and in different combinations, as depicted in Table 2, with 15 treatments: 7 were individually tested (Table 1), and eight were combined (Table 2).

Table 1. Individual treatments and dosages

Treatment	Dosage*
	25 mL + 22.5 gr
Bacillus sp.+ Humic Acid [B+HA]	45 mL + 22.5 gr
$Ca(NO_3)_2$ + Humic Acid [$Ca(NO_3)_2$+HA]	450 gr + 22.5 gr
$CaCO_3$ + Humic Acid [$CaCO_3$+HA]	10 kg + 22.5 gr
Trichoderma spp. + *Bacillus* sp.+ Humic Acid [T+B+HA]	25 mL + 45 mL + 22.5 gr
Trichoderma spp. + *Bacillus* sp.+ Humic Acid + Elicitor [T+B+HA+E]	25 mL + 45 mL + 22.5 gr + 45 mL
Trichoderma spp. + *Bacillus* sp.+ Humic Acid + Elicitor + $Ca(NO_3)_2$ [T+B+HA+E+$Ca(NO_3)_2$]	25 mL + 45 mL + 22.5 gr + 45 mL + 450 gr
Trichoderma spp. + *Bacillus* sp.+ Humic Acid + Elicitor + $Ca(NO_3)_2$ + $CaCO_3$ [T+B+HA+E+$Ca(NO_3)_2$+$CaCO_3$]	25 mL + 45 mL + 22.5 gr + 45 mL + 450 gr + 10 kg

Table 2. Combined treatments and dosages

Treatment	Source/Company	Dosage*
Control	-	No additional treatment/ farm management
$CaCO_3$	Carbonato de Calcio (Disensa, Ecuador)	10 kg
$Ca(NO_3)_2$	Fernical (Fertisa, Ecuador)	450 gr
Trichoderma spp. -T	Trichoplus (Microtech Services, Ecuador)	25 mL
Bacillus sp. -B	Linor (Microtech Services, Ecuador)	45 mL
Humic Acid-HA	Brumik (Bioraiz, Ecuador)	22,5 gr
Elicitor-E	Defenseplus (Bioraiz, Ecuador)	45 mL

All treatments were applied during the rainy and dry seasons of the year 2018. After harvesting the experiment during the rainy season, new plants were sown in the same area as previously evaluated. Therefore, the products' application was repeated in the same area in the same manner as the previous cycle. Three response variables were determined: Weight of Lump (kg), Diameter of Lump (cm), and Root Weight (kg).

Frequency and Period of application

All experiments were performed in the year 2018. During the rainy season, two applications were carried out in weeks 5 and 11. Harvesting was performed in week 18. In the dry season, the initial application was carried out in week 23, and the second application in week 28. Harvest was performed in week 32. The temperature, radiation, and precipitation were recorded once per week are presented in Fig.1.

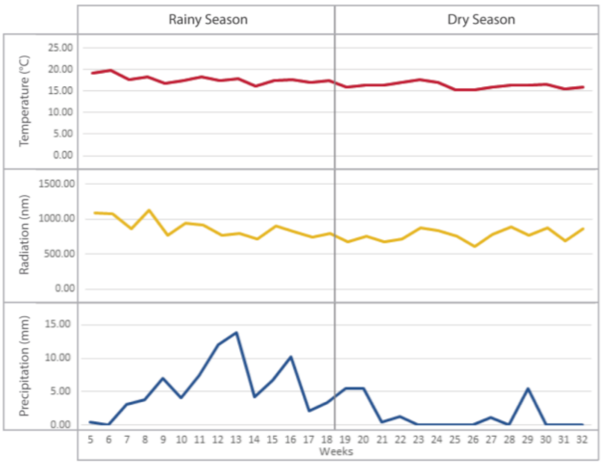

Fig. 1. Average temperature, radiation, and precipitation of the rainy season (weeks 5 to 18) and dry season (weeks 19 to 32) during broccoli production in Cotopaxi, Ecuador (Year 2018).

Statistical analysis

A completely randomized experimental design was carried out in plots of 25 m^2 for individual or combine treatments. Only inner plants per plot were used in the analysis to avoid the border effect. The analysis was performed with SPSS software within each harvest and between plots with an analysis of variance (ANOVA) + Tuckey test with 95% confidence. Ten (10) plants were taken randomly from each plot immediately after harvesting for analysis. For all treatments, three plots per treatment were analyzed per season organized in a complete randomized design.

RESULTS

Field selection and root damage evaluation

The experiments were carried out in a broccoli field where it was previously observed that plants had symptoms of *P. brassicae* (90% infestation). This field was selected for these reasons for further assays. Ten plant roots coming from the infested field were analyzed (Photo.1), and the morphology of the diseased showed significant malformations that affected the root system, which was observed better under the microscope, noticing cell damage differences compared with the healthy plant roots (control; Fig. 2).

Fig. 2. (a) Healthy plant root system, (b) Healthy plant root tissue, microscopy at 100X, (c) Infected plant root morphology, (d) Infected plant root tissue microscopy at 100X.

Single treatments evaluation

In terms of lump weight (kg), it had been found significant differences both when assessed individually and in combined treatments during the two seasons (rainy and dry; Fig.3). The first observation was the difference in total production (lump weight). The rainy season showed lower total production than the dry season (around 17% difference). This could be due to nutrient leakage and/or higher disease pressure, leading to total broccoli production changes. In the dry season, the lump weight was significantly different between $CaCO_3$ treatment and the other individual treatments, but with lump diameter and root weight parameters were no significant (Fig. 3).

The application of $CaCO_3$ in the dry season gave a significantly higher lump weight than the control treatment. With this treatment, the average lump weight was 0.60 kg, with an average lump diameter of approximately 22 cm and an average root weight of 0.20 kg. It was observed that in the dry season, the treatment with $CaCO_3$ increased the lump weight significantly. Thus, the same fertilizer in two different situations will not necessarily perform in the same manner, so it is essential to consider the experiment's environmental conditions.

According to Dixon (2010), calcium provides a long-term boost of soil microbial activity and offers a rapid series of crop growth opportunities to improve during the growing season. Besides, Donald *et al.* (2004). Demonstrated that the use of calcium in broccoli improves from 40% to 64%, reducing the effects of *P.brassicae*.

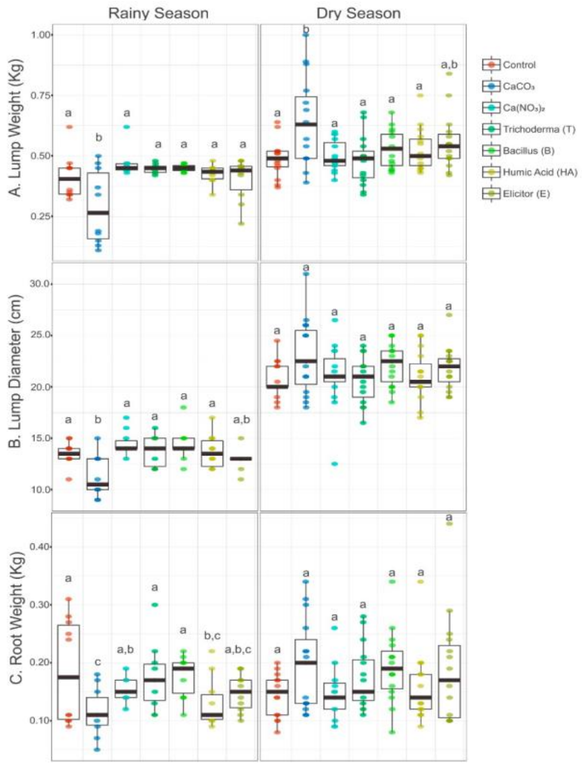

Figure 3. Evaluation of lump weight, lump diameter and root weight after the individual application of biological and calcium-based fertilizers during rainy and dry season.

In the rainy season, no improvement was observed when the treatments were applied individually, except when $CaCO_3$ was applied (Fig. 3). The lump weight, diameter, and root weight with the application of $CaCO_3$ gave significantly smaller values than its control during the rainy season; thus, the treatment reduced the yield, size of the lump, and root system of broccoli. Therefore, it was essential to test the combined treatments to find different responses and interactions.

Evaluation of the combined treatments

In the rainy season, for lump weight, there was a significant difference between the combined treatment of $CaCO_3$ + ($Ca(NO_3)_2$) + T+ B+ HA+ E versus the control treatment (Fig. 4). The average lump weight was approximately 0.62 kg for the combined treatment and 0.40 kg for the control treatment, for lump diameter, did not show any significant differences between combined treatments and the control. For the root weight, there was a significantly lower value between the combined treatments B + HA, $CaCO_3$+ HA, ($Ca(NO_3)_2$) + T+ B+ HA+ E and $CaCO_3$ + ($Ca(NO_3)_2$) + T+ B+ HA+ E versus its control treatment, giving as a result lower root biomass after the application (Fig. 4).

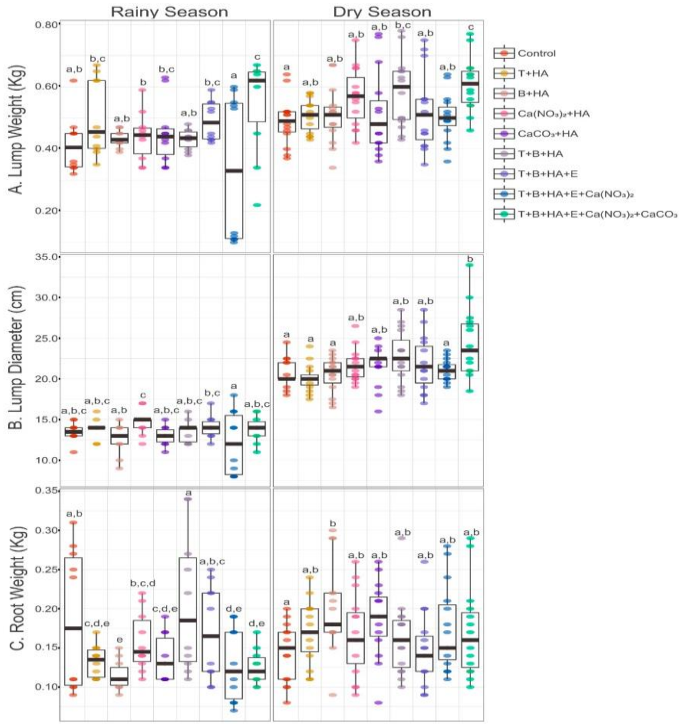

Fig. 4. Evaluation of lump weight, lump diameter, and root weight for combined treatments versus the control treatment during rainy and dry seasons.

In the dry season, for lump weight, there was a significant difference between combined treatment with $CaCO_3$ + ($Ca(NO_3)_2$) + T+ B+ HA+ E versus its control treatment. The average lump weight was approximately 0.62 kg for the combined treatment and 0.48 kg for the control treatment. There was significant difference for lump diameter between $CaCO_3$ + ($Ca(NO_3)_2$) + T+ B+ HA+ E versus the control treatment. The average lump diameter was 24 cm between the combined treatment and 20 cm for the control treatment.

Opposite results were obtained in the rainy season when root weight was evaluated, and it was a significantly higher difference between the B + HA (*Bacillus* sp.+ Humic Acid) treatment versus the control treatment. The average root weight was approximately 0.17 kg for the B + HA treatment and 0.15 kg for the control treatment, giving higher biomass when the B + HA was applied.

Taking all together, it was found that application of the combined treatments of $CaCO_3$ + $(Ca(NO_3)_2)$ T+ B+ HA+ E increased consistently lump weight and lump diameter in two continuous seasons tested in a field infested with *P. brassicae* (Fig.4). As already mentioned, the individual $CaCO_3$ application had variable effects and responses in the broccoli plants depending on the season.

DISCUSSION

In other studies, applications of *Trichoderma* spp. increase broccoli biomass, giving higher lump weight, lump diameter, and root weight. According to Camargo-Cepeda and Ávila (2013), *Trichoderma* spp. positively inhibited pathogen growth. However, in our study, no positives effects were observed when *Trichoderma* was applied as single treatments to broccoli plants. This could be due to differences in strains and environmental conditions. Another explanation could be that some studies reported some effects against *P. brassicae* however, they are performed only *invitro* and not in field conditions where the environment and doses might change the effectivity (Herrera-Romero *et al.*, 2017).

In addition, Xing-Yu *et al.* (2013) stated that *Bacillus* sp. showed biocontrol activity against broccoli pathogens and played a fundamental role in promoting the growth of the plant since it also has positive effects in root and lumps for the control to *P. brassicae.* However, in our study, no significant differences were observed in single treatments. Only effects in the root system were found when it was applied together with HA.

It has been reported that Humic Acids affected the growth and weight not only of roots but also of stems and inflorescence, with growth stimulation in some cases of 25% (Oliver, 2009). However, in our study, no significant effect was observed in single treatments with HA, only when included in combined treatments.

Elicitors could increase cellulose microfibrils that increase the lignification of cell walls, leading to the synthesis of defense proteins related to pathogenesis and the hypersensitivity response to the *P. brassicae* (Mogollón and Castaño, 2011). The impact of elicitors on lump weight was not proven before; however, Mogollón and Castaño (2011), argued that the elicitors could generate a control to this pathogen. In our study, no significant effect was observed in single treatments, as before. Only effects were found when included in combined treatments.

The best treatment found in our study was T+ B+ HA+ E+ $(Ca(NO_3)_2)$ + $CaCO_3$. Combining these compounds work synergistically, increasing the lump's weight and the diameter of the lump in the dry season. In the future, efforts should continue looking for alternatives that are friendly to the environment and help control *P. brassicae* minimizing the use of agrochemicals.

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