*PHIALOMYCES MACROSPORUS* REDUCES *CERCOSPORA COFFEICOLA* SURVIVAL ON SYMPTOMATIC COFFEE LEAVES

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**Abstract**

Saprobe fungi and necrotrophic pathogens share a same niche within crop stubble and the search for fungi non-pathogenic to plants that are able to displace the plant pathogens from its overwintering substrate contributes to the disease management. Brown eye spot (*Cercospora coffeicola*) is among the most important coffee diseases, it is caused by a necrotrophic pathogen that has decaying leaves as its major source of inoculum. We have screened saprobe fungi for the ability to reduce *C. coffeicola* sporulation and viability and determined the possible mechanisms involved in the observed biocontrol. A selected saprobe fungus, *Phialomyces macrosporus*, reduced the pathogen’s viability by 40% both *in vitro* and *in vivo*. The fungus acts through antibiosis and competition for nutrients. It produced both volatile and non-volatile compounds that inhibited *C. coffeicola* growth, sporulation and viability. It also produced the tissue maceration enzyme (polygalacturonase), which reduces the pathogen both in detached leaves or in planta. The reduction in the fungal viability either by the saprobe fungus or its polygalacturonase-fraction supernatant resulted in the reduction of the disease rate. Therefore, *P. macrosporus* is a potential microbial agent that can be used in an integrated management of brown eye spot through the reduction of the initial inoculum of the pathogen that survives and builds up in infected leaves.

**Keywords:** *Coffea arabica*, competition, biological control, brown eye spot, antibiosis

*PHIALOMYCES MACROSPORUS* REDUZ A SOBREVIVÊNCIA DE *CERCOSPORA COFFEICOLA* EM FOLHAS DE CAFÉ SINTOMÁTICAS

Resumo: *Cercospora coffeicola* (LFP 37) pode sobreviver como fungo saprófita em restos culturais de café, o que representa o inóculo inicial para novas infecções em folhas e frutos. Fungos sapróbios têm potencial em reduzir a sobrevivência de patógenos necrotróficos podendo atuar através da competição de nutrientes, micoparasitismo, antibiose e indução de resistência. Este estudo avaliou a capacidade de fungos sapróbios em inibir o desenvolvimento de LFP 37 em restos culturais de café, bem como os mecanismos envolvidos no biocontrole. O fungo sapróbio mais promissor foi selecionado, e, através de um ensaio *in vitro,* avaliado quanto a sua capacidade em sobreviver sob condições de stress hídrico e manter seu potencial antagônico. Foram analisados os mecanismos de biocontrole e a capacidade do sapróbio em acelerar o processo de degradação de restos culturais, sendo este avaliado através da quantificação de enzimas pectinolíticas. Além disso, por meio de ensaios *in vitro* e *in vivo* também foram avaliadas as contribuições de cada estrutura fúngica em reduzir o número de conídios do patógeno e acelerar a degradação das lesões. O fungo *P. macrosporus* foi o mais eficiente em reduzir a viabilidade do patógeno em restos culturais. Este fungo reduziu a viabilidade dos conídios de LFP 37 em 40%. O controle foi eficiente independentemente do stress hídrico imposto e, a produção de substâncias antimicrobianas foi o principal modo de ação utilizado pelo sapróbio. Pela primeira vez, foi apresentada uma opção de controle biológico para a mancha de olho pardo. O fungo sapróbio *P. macrosporus* foi capaz de controlar LFP 37 em restos culturais, o que resulta em um novo potencial de controle curativo a partir da utilização de um fungo sapróbio.

**Termos para a indexação:** *Coffea arabica*, competição, controle biológico, macha de olho pardo, antibiose

1. **Introduction**

Coffee (*Coffea arabica* L.) is an important commodity worldwide. However, diseases are responsible for many losses in this crop, such as brown eye spot (BES), caused by the necrotrophic fungus *Cercospora coffeicola* Berkeley & Cooke. The fungus is considered one of the most important coffee diseases and infects both leaves and berries, causing yield losses of approximately 50% (Zambolim et al., 2005).

Although effective fungicide-based alternatives are available for the disease management (Patricio et al., 2008), it should not be the exclusive tool but part of the integrated brown eye spot management such as plant nutrition (Cardoso et al., 2013) and biological control (Sirinunta and Akarapsan, 2015).

Considering conidia of *C. coffeicola* remains viable for up to nine months in coffee stubble (Zhang and Bradley, 2014) until favourable conditions for germination, the reduction of the initial inoculum should be taken into account as a strategy for the integrated management of the disease and this can be achieved by compost- accelerating products which encompasses both fast-growing fungi and cell wall degrading enzymes (Bellotte et al., 2009; Hauptman, 2014).

Because *C. coffeicola* is a necrotrophic fungus, its suppression may occur through a combination of mechanisms, such as the production of antimicrobial compounds, mycoparasitism and/or competition for nutrients. Competition for nutrients is likely to occur since hydrolytic enzymes produced by both pathogen and antagonist target the saprophytic survival on decaying leaves (Jurado et al., 2015; Alvarez-Rodriguez et al., 2016). Among these enzymes are pectinases, which are composed by an heterogeneous group of enzymes that degrades pectin (Falmy et al., 2008).

One such pectinase producers group are saprobic fungi. They play a significant role in the self- maintenance of a balanced ecosystem, mostly through the decomposition of organic matter, utilising a system of digestive enzymes that are secreted from the cells to the environment (Lin et al., 2015). Furthermore, they can withstand sudden variations in temperature and humidity and sustain biological control in such environment, which turn them promising biological control agents (Köhl et al., 1995). Currently, saprobic fungi have been developed for coffee disease control (Oliveira et al., 2011). Therefore, we hypothesised that saprobe fungi might inhibit *C. coffeicola* growth in crop residues and its production antifungal compounds and cell-wall degrading enzyme may play a role in the pathogen displacement.

**2. Materials and methods**

**2.1. Culture condition**

*Cercospora coffeicola* strain(LFP 37)was obtained from the Culture Collection of *Cercospora coffeicola* at the Plant Pathology Department, Federal University of Lavras, Minas Gerais State, Brazil. LFP 37 was grown for 10 d on solid V8 medium (V8S) (200 mL of V8 juice, 800 mL of distilled water and 20 g of agar) at 25℃. Afterward, LFP 37 sporulation was induced using the mycelial mass drying technique with some modifications (Souza et al., 2005). The pathogen suspension was adjusted to 3×104 conidia mL-1.

Three saprobe fungal strains were used in this study: *Phialomyces macrospores* (0053/07), *Curvularia inaequalis* (0005/06),and *Curvularia eragrostidis* (0047/06), obtained from the Culture Collection of the Microorganisms from Bahia, State University of Feira de Santana, Bahia, Brazil.

The strains were grown for 7 d in a medium of carrot maize agar (CMA) at 27°C. After incubation, one disk (7 mm) containing conidia and mycelium was suspended in 100 mL of CMA liquid medium, grown in an orbital shaker at 120 rpm and 27°C. The suspension was shredded, homogenized in a blender, and adjusted to 3.4×107 CFU x mL−1. Antagonistic fungi were inoculated by spraying the leaves until runoff, unless otherwise stated.

**2.2. Coffee seedlings**

Coffee seeds (Cultivar Mundo Novo IAC 376-4) were purchased from Agricultural Research Company of Minas Gerais (EPAMIG) and were seeded in plastic trays containing white sand for pre-germination. Two-month old seedlings were transferred to polyethylene bags (250 x 90mm) containing 3: 1 soil and sand substrate together with the super simple fertilizers (300g) and N.P.K (900G) in the mixture. Plants were kept in a greenhouse at 25–28℃ during the entire experiment.

**2.3. Screening test**

Pathogen inoculation consisted of spraying LFP 37 on the abaxial side of the coffee leaves (7-month-old) until runoff. Dark wet plastic bags were placed during 3 d to simulate a moist micro-chamber. At 15 d after inoculation (dai), when BES symptoms appeared, the three abovementioned antagonists were sprayed onto the leaves until runoff. Soil Set® (0.75 mL x L-1 ) associated with compost Aid® (0.75 mL x L-1 ) were used as positive control, as described by Bellotte et al. (2009), while distilled water was applied as a negative control. After saprobe application, 10 leaves from each treatment were stored in plastic bags at 28℃ for 7 and 14 d. Conidia count and germination of LFP 37 were determined.

To determine the number of conidia, lesions of each leaf were excised and transferred to 10 mL tubes with 1 mL of distilled water with 300 µL of Tween 20% (v/v). Aliquots of each suspension were counted using a haemocytometer. For the viability spore test, 400µL of the suspension of each treatment was transferred to 90mm Petri dishes containing 15 mL of water-agar at 2% (w/v). The plates were incubated at 25ºC for 6 h, and the number of germinated spores was counted under light microscope at 40×. The conidia were considered germinated when the germ tube was equal to or greater than half of the spore length (Beckman and Payne, 1983).The experiment was performed twice, and the fungus with the highest potential to reduce pathogen survival was selected to continue the study. The experimental design was a completely randomized block in a factorial scheme with three blocks containing five plants in each treatment.

**2.4. Assessment of antagonist drought-tolerance**

Drought tolerance was assessed to study the ability of *P. macrosporus* to reduce LFP 37 survival in plant lesions under humidity variation to simulate field conditions (Köhl et al*.*, 1995). Fungal growth, plant cultivation, and fungal spray were performed as described above. Immediately after antagonist application, wetness was interrupted. Thereafter, 10 leaves per treatment were placed on two layers of sterile dry filter paper in open Petri dishes in laminar flow cabinet for 9 h. The leaves were incubated for two different periods of interrupted wetness (0 and 61 h) after the antagonist application. (Köhl et al.,1995). After the dry period, the filter paper was wetted with 2 mL of sterile tap water. Then, the Petri dishes were closed and ketp under the same conditions, as described above. After 7 d, pathogen conidia and germination were determined, as previously described. The experimental design was a completely randomized block design in a factorial scheme with three blocks and 10 leaves per plot.

**2.5. Antibiosis and volatile production test**

A method of measuring the direct activity of antagonists against pathogens was used in the *in vitro* antibiosis and volatile production test (Prasad et al., 2011). These assays were performed by cultivating the fungi on regular and bipartite Petri for antibiosis and volatile production, respectively. The putative ability of *P. macrosporus* to produce different molecules or enzyme toxic to the pathogen was assessed by the confrontation assay. Plates were incubated at 25°C and 12 h in the light for 5 d. The assay was evaluated by cultivating *P. macrosporus* on one side of the Petri dish and *C. coffeicola* on the opposite side. The fungi were cultivated on Potato Dextrose Agar (PDA, 39 g L-1), CMA and V8 media. The negative control contained only the pathogen in one side of the plates. The effect of molecules or enzyme toxic produced by *P. macrosporus* on *C. coffeicola* was quantified by comparing the mycelia growth and number of conidia. The experiment was distributed in a randomized block design with four replicates and was performed twice.

The production of fungitoxic volatiles compounds by *P. macrosporus* was evaluated by cultivating *P. macrosporus* in one side of a bipartite Petri and *C. coffeicola* in the opposite side. Plates were incubated at 25°C and 12 h in the light for 5 d. The fungi were cultivated PDA, CMA and V8S. The negative control contained only the pathogen in one side of the plates. The effect of fungitoxic volatiles compounds by *P. macrosporus* on *C. coffeicola* was quantified by comparing the mycelial growth, number of conidia and germination (%).The experiment was distributed in a randomized block design with four replicates and was performed twice.

**2.6. Pectinase production**

The ability to produce hydrolytic enzyme in solid medium was assessed by growing *P. macrosporus* on mineral medium (pH 7.2) (2.0 g L-1 KH2PO4; 7.0 g L-1 K2HPO4; 1.0 g L-1(NH4)2SO4; 1.0 g L-1 MgSO4.7H2O; yeast extract 0.06% (p/v); and 13 g L-1 agar). The medium was supplemented with 3 g L-1 citrus pectin and incubated at 27°C for 5 d. Agar discs (7 mm diameter) containing mycelia from the colonies were transferred to Mac McIlvaine buffer (0.2 M NaHPO4, 0.1 M citric acid, 13 g L-1 agar-pH 6.0) with 0.25% (w/v) citrus pectin and incubated at 40°C for 48 h. A solution of I2/KI (1 g of I2 and 5 g of KI in 330 mL of H2O) was added to detect the clearing zones.

**2.7. Fungal growth and Polygalacturonase (PG) activity**

The ability to produce PG in liquid media was evaluated by growing *P. macrosporus* on mineral medium (6.8 g K2HPO4; 3.8 g KH2PO4; 1.0 g (NH4)2SO4; 1.1 g MgSO4.7H2O; 0.6 g yeast extract, 3 g pectin 2% in one litter of distilled water). Erlenmeyer flasks (125 mL) containing 50 mL of the described medium were inoculated with one disk (7 mm) of mycelium and shaken at 150 rpm at 27°C to get a final spore concentration of 106 spores mL-1. The samples were collected every 24 h for 120 h. The PG activity was determined by mixing 1 mL of culture filtrate in 1 mL of substrate solution (100 mM sodium acetate buffer;[ pH 4.5]; 0.2 mM NaCl, 1.2% polygalacturonic acid [ w/v]) and incubated at 40ºC for 20 min . The reducing sugars released in the medium were measured by using the 3.5-dinitrosalicylic acid methods with galacturonic acid as a control. One PG unit was defined as micromoles of galacturonic acid released per minute per millilitre. The fungal growth was measured daily for 5 d, the mycelium was collected by filtration through nylon cloth under vacuum, the mycelium was dried at 60ºC for 48 h and it was weighed the dry mycelium mass contribution of each fungal structure to the decreased number of pathogen conidia and the lesion degradation.

To determine the ability of the saprobe to displace the pathogen from lesions, the contribution of each fungal-derived product or enzyme was evaluated *in vitro* and *in vivo*. For the *in vitro* assay, coffee leaf samples naturally infected by LFP 37were collected from field-grown fungicide-free plants. Leaf lesions were cut off and separated into blocks according to lesion sizes, which varied from five to eight injuries per treatment.

Initially, the number of conidia and half the initial weight of each lesion were determined. Subsequently, the lesion disks were sprayed with each treatment, which were saprobe conidia, enzyme polygalacturonase produced by the saprobe, conidia +supernatant of the saprobe, only the supernatant of the saprobe growth and water. In every case, the fungi were grown for the previously determined minimum time necessary for the maximum activity of polygalacturonase. After 7 d of incubation, the number of conidia of LFP 37was assessed to determine the contribution of each treatment in accelerating the deterioration of the lesions. The experimental design was a completely randomized block with four blocks containing five to eight excised leaf lesions per treatment.

An *in vivo* assay was conducted to verify the efficacy of the saprobe fungi. Coffee plants seedlings cv ‘Mundo Novo IAC 376-4’ were inoculated with LFP 37and from the onset of symptoms (25 dai), each fungal-derived product or enzyme mentioned above was sprayed onto the leaves until runoff. Then, 10 leaves from each treatment were used to determine the pathogen conidia count and initial germination. After 7 d of incubation, the conidia and final germination were assessed. The experimental design was a completely randomized block with four blocks containing five treatments. This experiment was repeated to check for consistency of results.

**2.8. Experimental design and statistical analysis**

Data from different experiments were tested for normality and submitted to an analysis of variance (ANOVA), Tukey and Scott-Knott multiple range tests (*P*=0.05). Data related to the Polygalacturonaseand dry mycelial weight of the fungus *P. macrosporus* were submitted to regression equations and subjected to a parallelism (*F*-test) test. The goodness of fit of the models was evaluated by coefficient of determination (R²). The data analysis was performed in the R software version 3.1.1.

1. **Results AND Discussion**

To the best of our knowledge, for the first time a biological control option for BES targeted its saprophytic phase and it was best achieved by the use of *P. macroscopus* (Figure 1). In the screening assay, there was no significant interaction between the treatments and the time when the experiment was carried out for either the conidia count (*P*=0.46) or the viability of LFP 37 conidia (*P*=0.14). Regarding LFP 37 conidial germination, these in vivo assays showed that the application of *P. macrosporus* to the injuries caused by the pathogen reduced the viability of LFP 37 by 40% in 7 d (*P*=0.0003) (Figure 1). This effect persisted for at least 14 d after application of the antagonist under study (Figure 1), showing that not only was saprobe able to reduce the viability of the pathogen, but this effect also occurs at the earliest considered time point (7 d after treatment). Odile-Mathieu and David Daniel (2006) studied isolates of *Microsphaeropsis ochracea* in the lesions of onion leaves during senescence and reported a reduction in the sporulation of *Botrytis squamosa* from 10 days after the application of the antagonist. In our study, the contribution of the reduced inoculum of Cercospora to the occurrence of BES in the field was not evaluated, but a similar study by Galletti et al (2008) showed that when *Trichoderma* sp. was applied to the leaves of sugar beet infected with *Cercospora beticola*, the viability of the pathogen spores decreased, resulting in a delay of the epidemic in the subsequent crop.

The performance of antagonists under field conditions is determined mainly by their ecological competence. Regardless water stress condition (*P*=0.82 and *P*=0.95), there was a reduction of conidial viability of up to 37.72%. This result is in agreement with Kohl et al. (1995), who showed suppression of sporulation of *Botrytis aclada* in dead onion tissue treated with the antagonist *Ulocladium atrum* in bioassays with repeated wet-dry-cycles. In addition to evaluating the potential of the saprobe to control the pathogen regardless of the stress to which it is exposed, which aims to determine the sustainability of the control technique, it is necessary to evaluate the action mechanisms of the biocontrol agent. One of the most common mechanisms of reduced survival of pathogens is antibiosis. Hiradate et al. (2002) observed a decrease in the survival of *Colletotrichum dematium* on mulberry leaves infected when treated with *Bacillus amyloliquefaciens*, confirming that the production of antifungal compounds was the primary mechanism of action that was used by the antagonists under study. For the antibiosis test, interaction was observed between medium and treatments (*P*=0.0090). A positive interaction was observed between culture on the media CMA (*P*=0.0018), V8 (*P*=≤0.001), and production of non-volatile compounds, resulting in greater pathogen control (Figures 2 and 3). Thus, it is suggested that such culture media induce the production of a greater amount or range of compounds with an inhibitory action of LFP 37. Similar results was obtained by Abe et al. (2015) who isolated a class of secondary metabolites, polyketides from the fungus *P. macrosporus*, which is known to provide survival advantages to microbial hosts. Some of the benefits could be the production of antibiotics, as reported by Zhang et al. (2015) who studied the antibiosis functions during interactions of *Trichoderma* ssp. with plant pathogenic *Rhizoctonia* and *Pythium*. The authors confirmed that polyketide positively correlated to the production of antibiotics.

The competition mechanism between microorganisms in a colonising necrotic area was tested by determining and quantifying pectinolytic enzymes. These enzymes degrade dead plant tissue and release nutrients for both the pathogen and saprobe. Thrane et al. (2000) monitored GFP-tagged *Trichoderma* spp. saprobes in cucumber plants infected with *Pythium ultimum* and found that the biocontrol isolates were able to take up the nutrients in the plant tissue more efficiently than was the pathogen, confirming that competition for nutrients is an important mechanism in this biocontrol pathosystem. Rodríguez et al. (2016) recently confirmed this hypothesis by determining that the niche overlap index between *P. macroscoporus* and *Colletotrichum gloeosporioides* was 100%, i.e., all nutrients the pathogen uses, the biocontrol agent also does and surviving in the necrotrophic tissue provoked by the pathogen.

Furthermore, in addition to externally colonising plants, one important feature of fungi is the colonization of live plant organs and exerting benefits to the plant over the long term (Barahona et al., 2011). Reports by Ahmad et al. (2003) showed that *P. macrosporus* colonizes coffee berries of robusta and arabica. Therefore, similar to *Cladosporium cladosporioides* isolates, *P. macrosporus* growth may acts as a barrier to the entry of other harmful fungi quality of coffee. Furthermore, *C. cladosporioides* is characterized as a saprophyte fungus and PG enzyme producer (Rezende et al., 2013).

In this study, we identified the production of PG on solid medium through the formation of the corresponding halo enzyme production. The average diameter of the formed halo was 0.93 cm. However, when we compared the *P. macrosporus* activity of PG (744.2685 U/mL) with microorganisms that have the ability to secrete large amounts of this protein, such as *Penicillium griseoroseum* (1600 U/mL), we cannot consider this saprobe a significant PG producer (Minussi et al. 1998). Although, we suggest a synergistic activity of this enzyme and antibiosis act on the pathogen displacement from the lesion hence in reduction of its survival on detached leaves and in vivo test. Further studies are necessary to investigate the role of PG in the observed biocontrol.

Contribution of each fungal structure to the decreased number of pathogen conidia and lesion degradation *in vitro* and *in vivo* assays were conducted to evaluate fungal structure. From the *in vitro* experiment, this supernatant was not effective in the degradation of lesion tissue in the short-term evaluation (7 d after treatment) (*P*=0.91), suggesting more cultivation time to achieve a significant decrease in weight of leaf and therefore treatment effectiveness. Additionally, there was no difference between the treatments for the number of conidia of LFP 37 (*P*=0.44). On the other hand, *P. macrosporus* consistently reduced LFP 37 conidia viability in detached leaves and in coffee plants. For the *in vivo* assay, not only the conidia of the saprobe fungus but also all of the fungal-derived product or enzyme reduced the germination rate by 40% (*P*=0.00003 and 0.00) (Figure 6). Since there was no significant difference between these treatments, either one could be used as a control, which gives more versatility to the use of this fungus as a future biological product. In addition, other authors have reported the use of the fungus *P. macrosporus* as a biocontrol agent in coffee diseases. Botrel and Medeiros (2013) observed a reduction in the severity of coffee halo blight of up to 72% when the seedlings were previously treated with the fungus *P. macrosporus*. Rodríguez et al. (2016) observed similar results in the control of *C. gloeosporioides* in coffee seedlings treated with *P. macrosporus*.

1. **Conclusions**

In summary, *P. macrosporus* was the most effective to reduce pathogen viability in crop residues. This effect is regardless of the wetness period and act mainly by producing antimicrobial substances. For the first time, a biological control option for BES is presented and the saprobe fungus *P. macrosporus* was able to control LFP 37 in crop debris, which results in a novel curative potential of a saprobe fungus for coffee BES.

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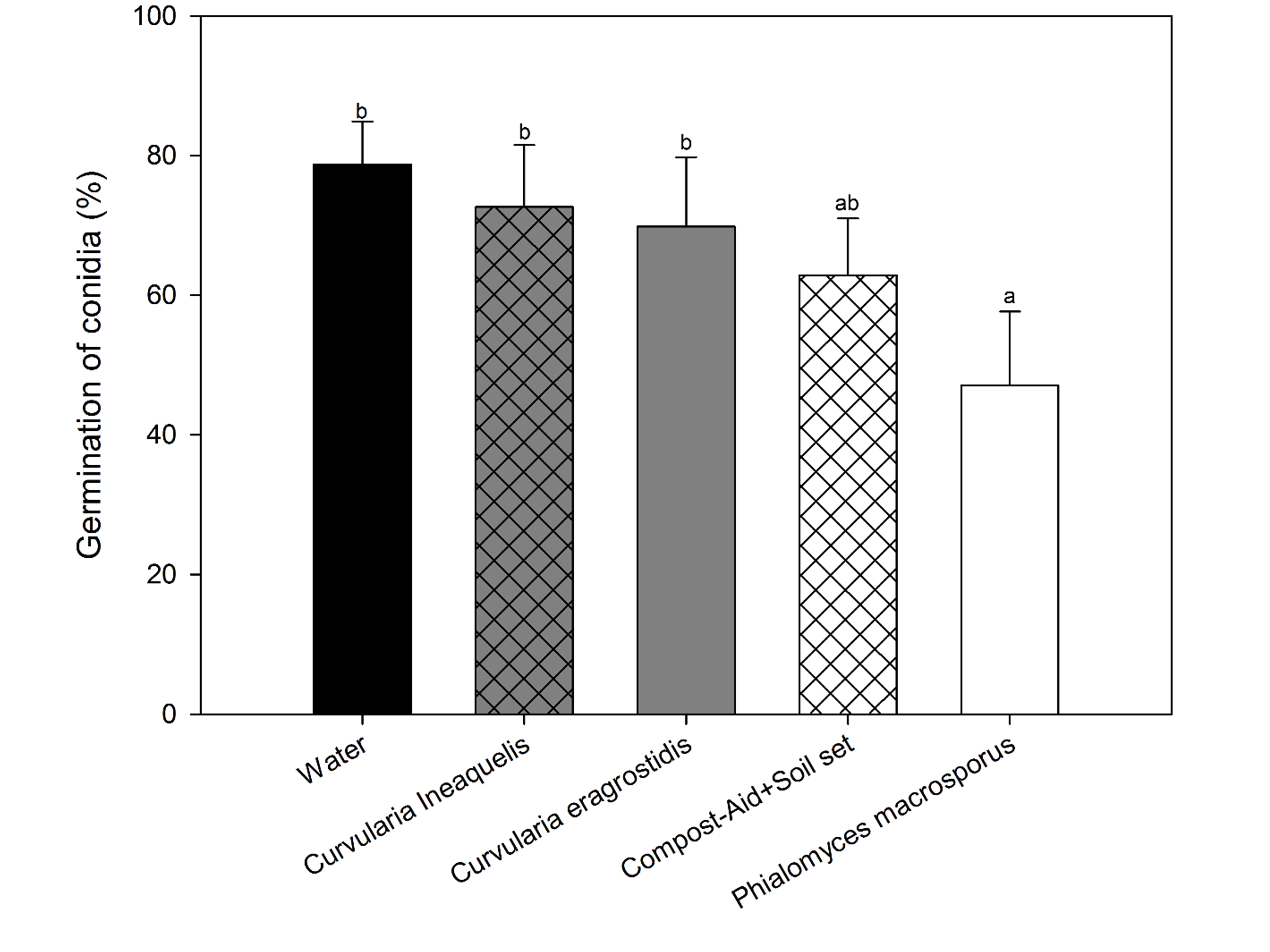
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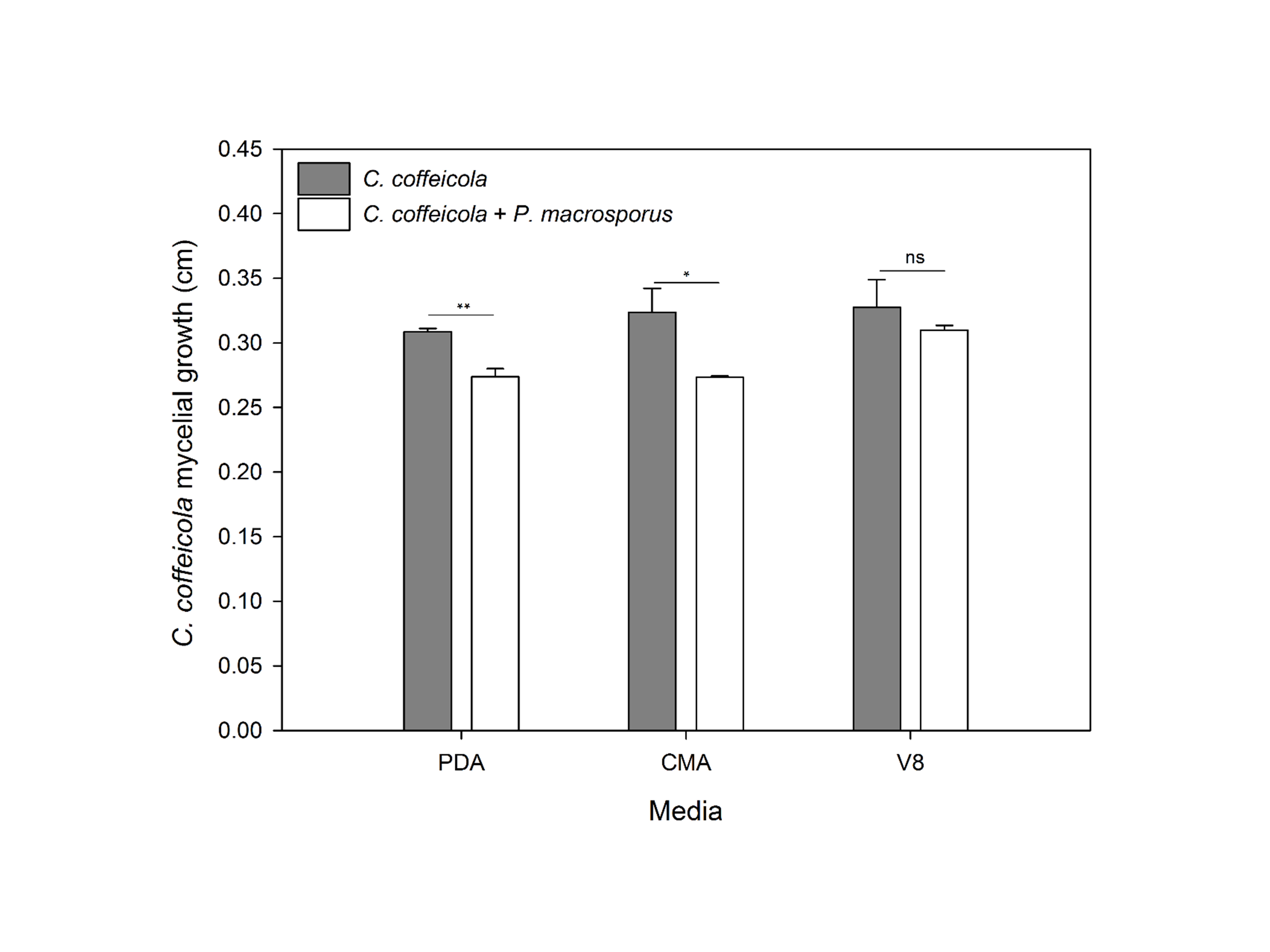
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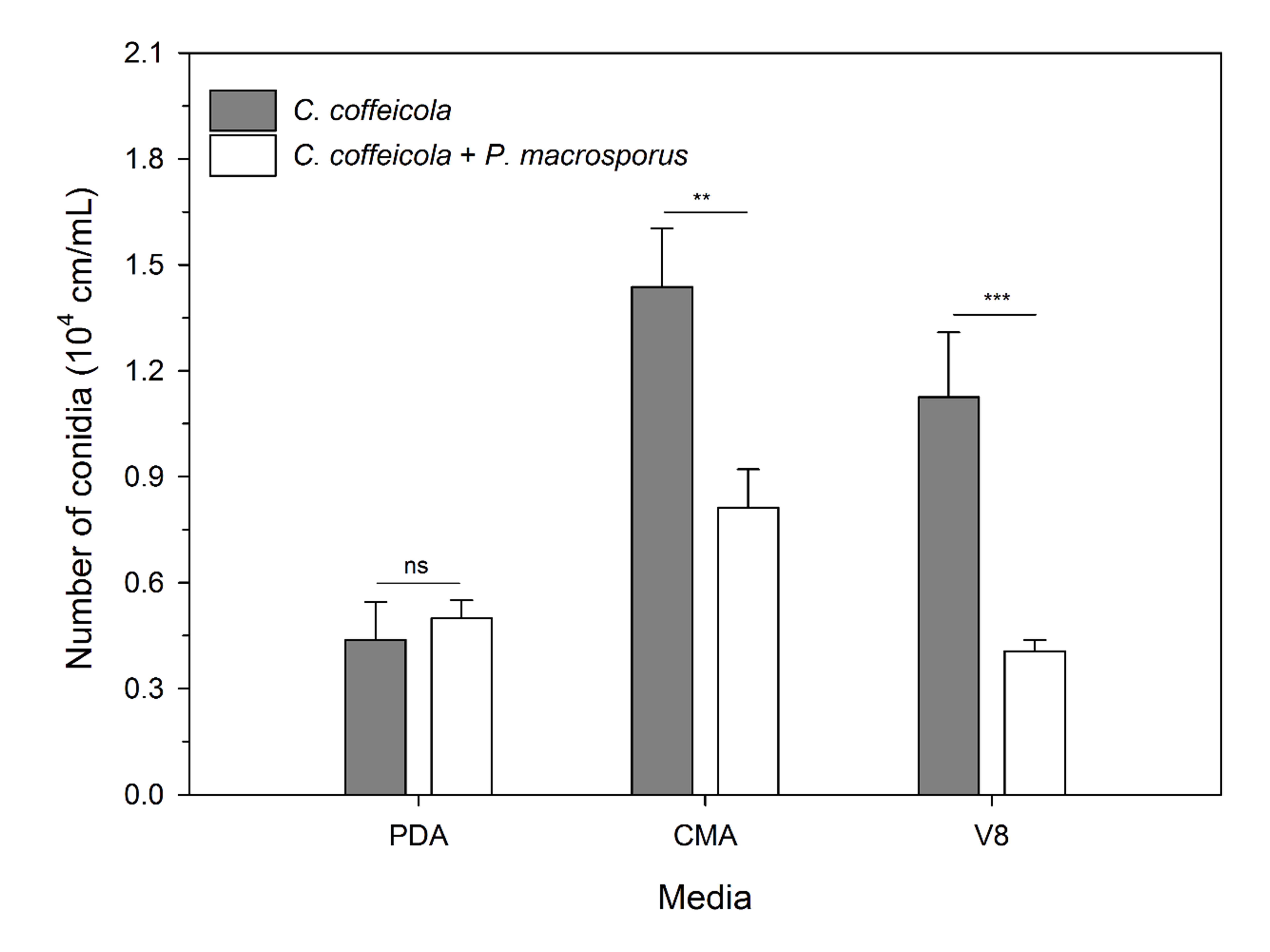
**FIGURES**

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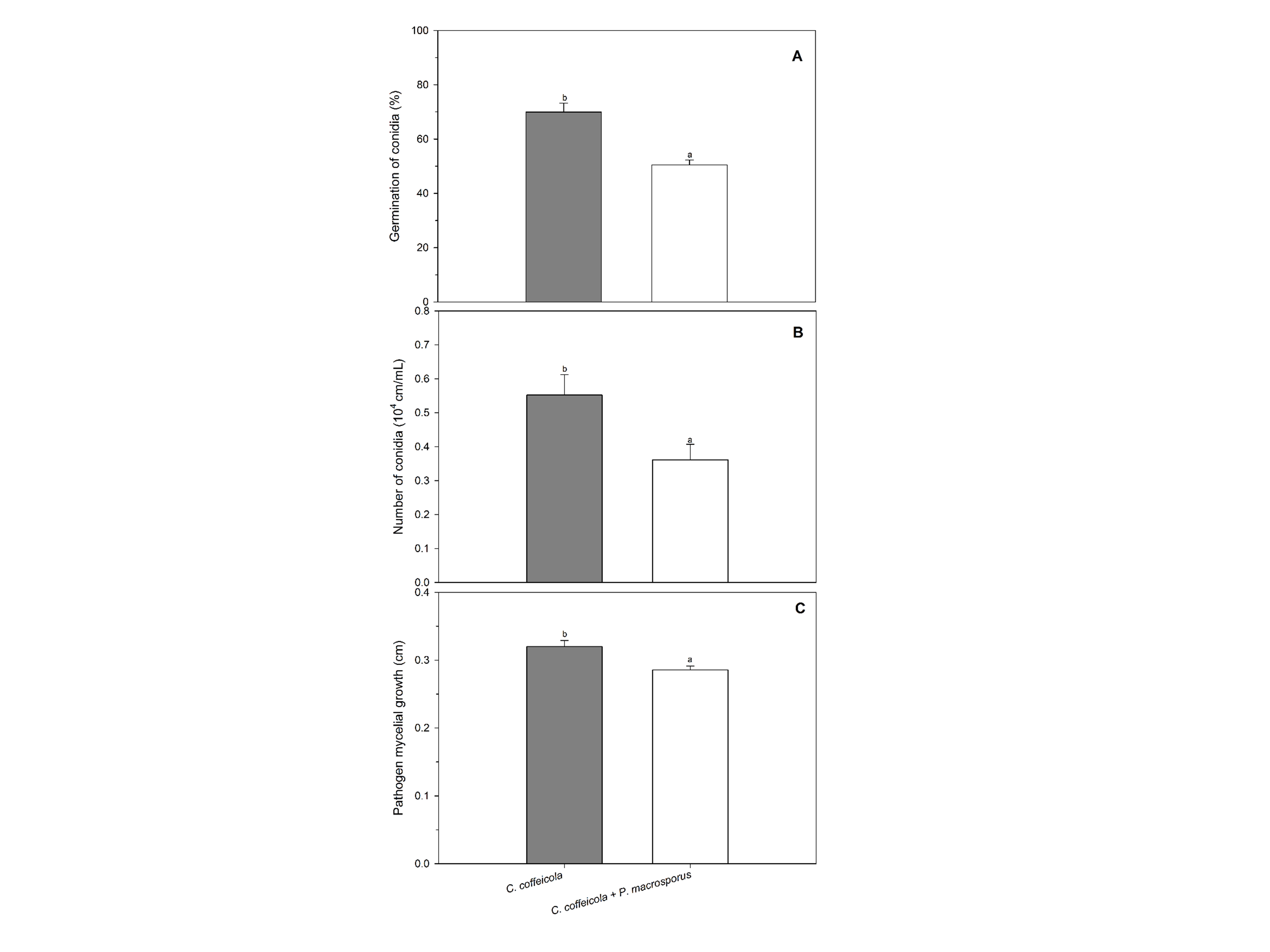
**Figure 1** Germination of *Cercospora coffeicola* conidia evaluated at 0, 7, and 14 days after the application of saprobe fungi. Bars with the same letter are similar at the 5% level according to Tukey multiple range test. CV=24.85%. The line on each bar represents ±SE.

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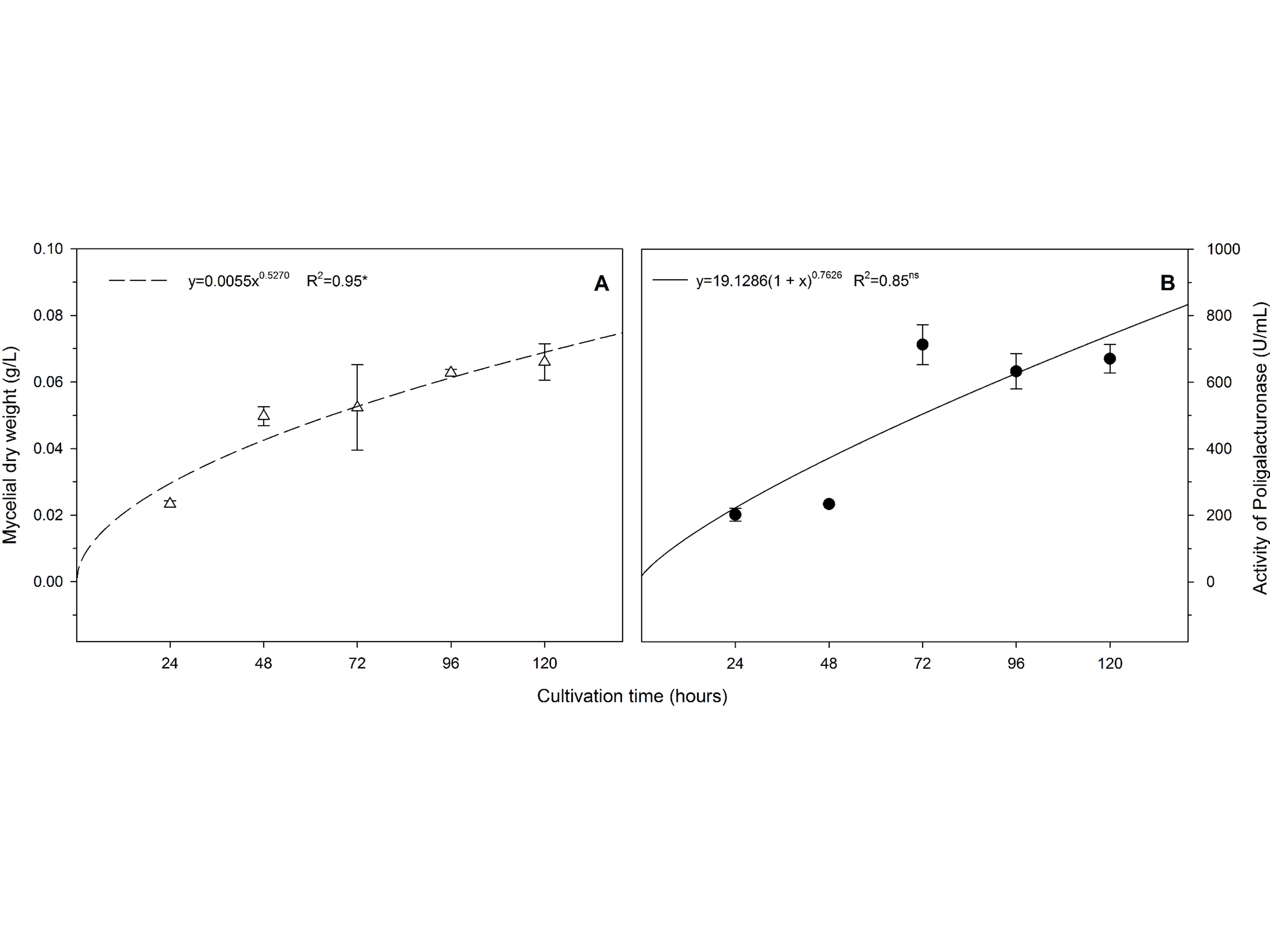
**Figure 2.** *C. coffeicola* mycelial growth with and without *P. macrosporus* on the media PDA, CMA, and V8. Related graphic analysis of the unfolding of the treatments (*C. coffeicola* and *C. coffeicola* + *P. macrosporus*) in each level of the factor of culture medium. Means with the same letter are similar according to the Student t test \*\*\*(*P*≤0.001). nsNon significant by the Student t test. CV=10.60%. Each bar represents the mean of four replicates (Petri dishes) per treatment per culture medium.

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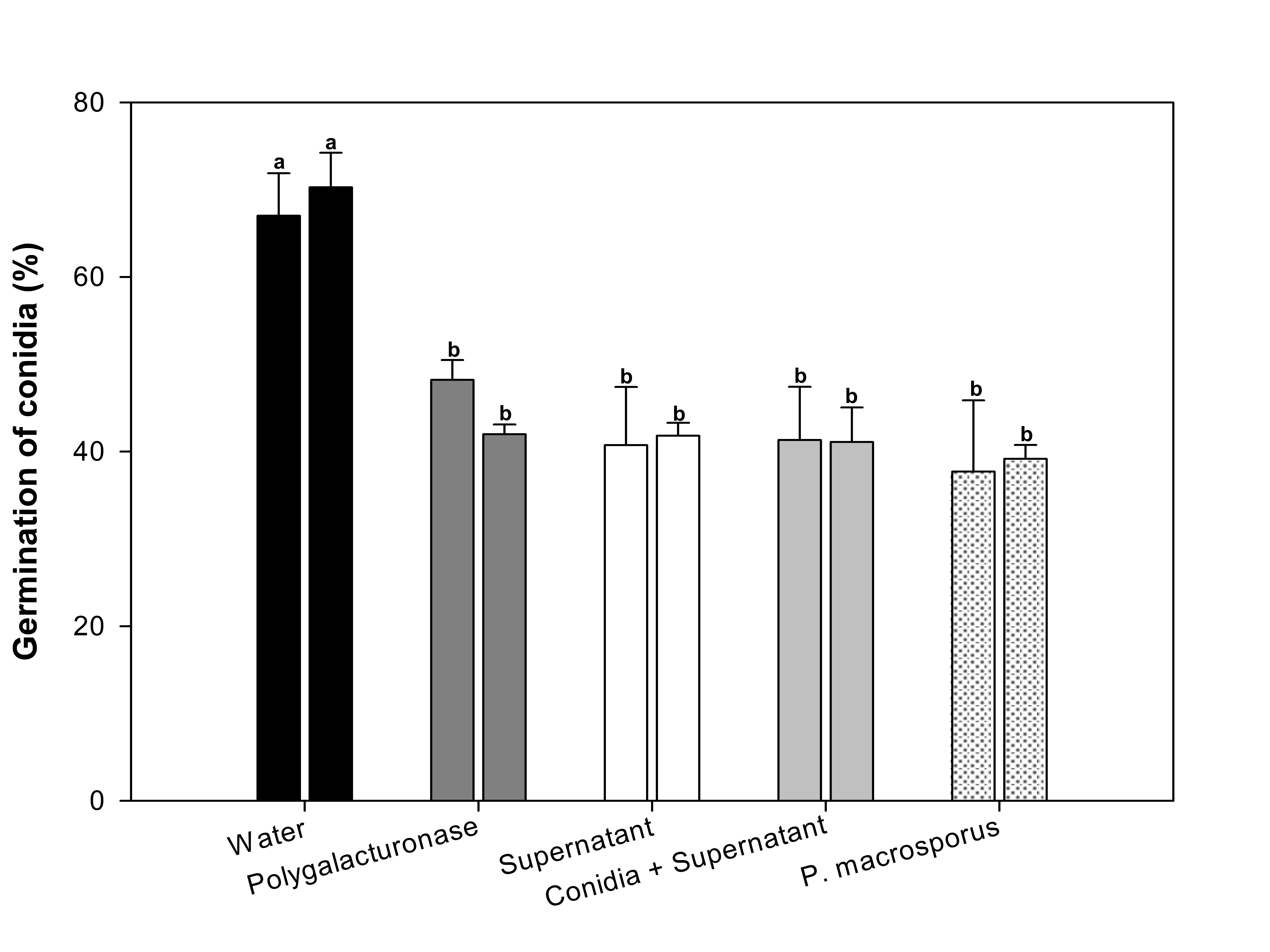
**Figure 3.** Number of conidia of the fungus *C. coffeicola* with and without *P. macrosporus* on the media PDA, CMA, and V8. Graphic analysis of the unfolding of the treatments (*C. coffeicola* and *C. coffeicola* + *P. macrosporus*) within each level of the factor of culture medium. The means written with a common letter do not differ significantly according to the Student t test \*\*(*P*≤0.01) or \*\*\*(*P*≤0.001). nsNon significant by the Student t test. CV=30.66%. Each bar represents the mean of four replicates (Petri dishes) per treatment per culture medium.

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**Figure 4.** Effect of volatile production on mycelium growth, germination and sporulation of *C. coffeicola*. Graphs A, B, and C represent the conidial germination (%), number of conidia, and mycelial growth (cm) of *C. coffeicola* without and with *P. macrosporus*, respectively. The bars with the same letter are similar at the 5% level according to the Student t test (*P*≤0.05). CV = 13.23%, 39.11%, and 7.80% for graphs A, B, and C, respectively. The line on each bar represents ±SE.

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**Figure 5.** The regression lines were used to describe the growth curve and activity of PG fungus *P. macrosporus*. (A) Dry mycelial weight. (B) PG activity in the culture supernatant. The line on each bar represents ±SE. \*Angular coefficients differ significantly by *F*-test.

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**Figure 6** Germination of *C. coffeicola* conidia after the application of *P. macrosporus* conidia at 107mL-1, the conidia+supernatant, supernatant alone, the polygalacturonase enriched supernantant or water. The graph represents two assays conducted in different periods. The bars with the same letter are similar at the 5% level according to Scott-Knott’s multiple range test. CV=14.48% and 12.01%, respectively. The line on each bar represents ±SE.