

Inhibition of the fungus *Colletotrichum lindemuthianum* in the presence of different potassium sorbate concentrations

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Abstract

One of the major problems of *in vitro* plant cultivation is caused by contaminations such as fungal, which represent great losses for plant cultivation laboratories. In view of the problem about contamination and gaps in efficient disinfection protocols, the present study aimed to evaluate the inhibition of the fungus *Colletotrichum lindemuthianum* in the presence of the potassium sorbate food preservative at different concentrations (0 g L⁻¹; 0.076 g. L⁻¹; 0.15 g. L⁻¹; 0.30 g. L⁻¹; 0.45 g. L⁻¹; 0.60 g. L⁻¹ and 0.75 g. L⁻¹). The analyses of the Percentage of Micelial Growth Inhibition (ICP) and the growth at 24 and 72 hours at different concentrations were quantified. It was observed that Potassium Sorbate was effective in the analysis of ICP at different concentrations in relation to control, with greater effectiveness for T4 to T7. The highest percentages of inhibition (T5 to T7) presenting an inhibition rate of mean mycelial growth of 17.5 % in relation to the control for the 72h period, indicating, therefore, that with further studies this preservative can be used to combat fungal microorganisms *in vitro* culture.

Keywords: Inhibition. Microorganism. In vitro contamination. Fungal inhibition. Mycelial growth. Food preservative.

Introduction

The development and improvement of plant manipulation techniques *in vitro* contributes to the production of plant species on a large scale and at all times of the year (LEIFERT; CASSELLS, 2003). These techniques consist of the production of clones of a plant from an explant, and the complete regeneration of a fragment to a complete plant is based on the principle of cellular totipotency, and at the end, a plant will be obtained that will present the same genotypic constitution as the one that gave rise to it (TORRES *et al.*, 2000).

Despite numerous advantages found in large-scale *in vitro* production, some problems have not yet been fully or safely solved, such as contamination, which cause large losses of plant material and which are possibly the main problems encountered in tissue culture (GRATTAPAGLIA; MACHADO, 1998; LEIFERT MORRIS; WAITES, 1994; CASSELLS, 2000b). Contaminations can occur by fungi, yeasts, bacteria, viruses, and also by the operator who manipulates plant material (GEORGE, 1993; CASSELLS, 2000a). Thus, to inhibit or minimize the problem resulting from contamination, numerous disinfection procedures and protocols are used in *in vitro* micropropagation laboratories (YEPES; ALDWINCKLE, 1994; PIERIK, 1997; VIANA *et al.*, 1997; PASQUAL, 2001; HIRATA; MANCINI-FILHO, 2002; COLOMBO *et al.*, 2004; EMMANUEL *et al.*, 2004).

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A possibility in view of the problem of contamination is the use of food preservatives such as Potassium Sorbate ($C_6H_7O_2K$), added to the culture medium, considering that it is already widely used in various foods, cosmetics, and pharmaceuticals for the containment of decaying microorganisms such as fungi and their spores, bacteria, and yeasts (OLIVIER *et al.*, 1998; SUHR; NIELSEN, 2004; GUYNOT *et al.*, 2005; BRAZIL, 1999; ESFANDIARI *et al.*, 2013), being considered a product with dependent dose action (OLIVIER *et al.*, 1998; FAGUNDES *et al.*, 2013). Has decomposition at temperatures above 270 °C, which favors its use in culture media due to its previous sterilization (SOFOS, 1995). Its inhibitory action has been attributed to the inhibition of enzymes, and the substance may accumulate in the cytoplasmic membrane of microorganisms, interfering in the transport of substrates and oxidative phosphorylation, resulting in mycelial non-growth and its sporulation (FREESE, SHEU; GALLIERS 1973; SOFOS, 1995; FALLIK *et al.*, 1997; PALMER, HORST; LANGHANS 1997; HEYDARYINIA, VEISSI; SADADI, 2011). Some studies have demonstrated its efficacy in plantations, that is, in the *in vivo* condition (DELIOPOULOS, KETTLEWELL; HARE 2010; MECTEAU; ARUL; TWEDDELL, 2002), and studies using Potassium Sorbate or its derivatives as inhibitors of microorganisms *in vitro* are scarce. Due to this gap, this article aimed to evaluate the inhibition of a fungus (*Colletotrichum lindemuthianum* Sacc. & Magnus) Briosi & Cavara 1889 in the presence of different concentrations of potassium sorbate, with a view to its use *in vitro* plant cultivation.

Material and methods

Choice of the potassium sorbate food preservative

The choice of this food preservative was due to the fact that it is already used in the food, cosmetic and pharmaceutical area, has easy to obtain, it has an affordable cost and it has a decomposition temperature above 270 °C, and thus remains stable during the autoclaving process, as well as presenting a versatility as to the broad spectrum of microorganisms whose growth is inhibited (BRAZIL, 1999; GUYNOT *et al.*, 2005; ESFANDIARI *et al.*, 2013), according to the range of microorganisms that occur *in vitro*, and also, for presenting low levels of human toxicity, with a median lethal dose of 500 g in an adult (OLIVIER *et al.*, 1998).

Isolation, choice, and culture medium of the fungus *C. lindemuthianum*

The phytopathogenic fungus *C. lindemuthianum* was isolated from lesions in the bean (*Phaseolus vulgaris* L.). Small sterilized fragments of the lesions were collected and inoculated in the center of plates with Potato-Dextrose-Agar (BDA) medium, 20 mL, and kept in the dark at 25 ± 2 °C for 72 hours. After isolation and growth in plaques, the fungus was used in the treatments.

The choice of this fungus was due to its ease of isolation and cultivation, as well as the literature showing that fungi of the genus *Colletotrichum* infect various cultures and some are causing anthracnose disease (MUNCH *et al.*, 2008; DAMM *et al.*, 2010). In addition to infections that can be observed in the *in vivo* condition, such fungi can also infect explants when inoculated *in vitro* in the most different plant species, making this fungus quite recurrent *in vitro* cultivation (HORNER; AUGUSTIN; FORCELINI 2001; MARTIN *et al.*, 2003), taking advantage of the nutritional environment for its development (KRISHNA; SINGH, 2007; ANDRADE *et al.*, 2008).

Effect of inhibition of mycelial growth of the fungus *C. lindemuthianum*

With the aid of a 5 mm hole punch, uniform fragments of 5 mm diameter of BDA medium were obtained with mycelium of the fungus growing after 72 h of the previously described isolation (MENTEN *et al.*, 1976). These culture medium discs containing mycelium were inserted in the center of three plates for each of the treatments, as well as in three plates without the presence of potassium sorbate (control).

After inoculation of the discs, the plates remained in the growth room at 25 ± 2 °C, with a photoperiod of 12 h for a total time of 72 h, considering that above this time the control plate reaches the maximum growth margin within the culture medium. The mean radial diameter of the fungus growth was performed every 24 hours. The capacity in Percentage of Mycelial Growth Inhibition (ICP) was verified through the equation described by Edgington, Khew and Barron, (1971) with modifications suggested by Menten *et al.* (1976).

The radial growth of the fungus was always measured on the same axis during the analyzed period, taking as a reference point the center of the sample inoculated at time zero. It was inferred from the sampling center to the edge of mycelial growth, every day at the same time. The center of the 5 mm sample disc was demarcated, in addition, the same position was always demarcated for the sampling of mycelial growth values to the edge of the mycelium over the test time.

Potassium sorbate concentrations

Culture media were made with 6 different concentrations of Potassium Sorbate of the Adicel brand, in addition to control. Thus, the following treatments were obtained: T1: Control; T2: 0.076 g L⁻¹; T3: 0.15 g L⁻¹; T4: 0.30 g L⁻¹; T5: 0.45 g L⁻¹; T6: 0.60 g L⁻¹, and T7: 0.75 g L⁻¹. These concentrations were based on previous own studies that used the same food preservative *in vitro* cultivation, and the results showed that concentrations above 0.75 g L⁻¹ caused complete oxidation of the explant and subsequent death, and thus its use would be unfeasible to contain microorganisms for cultivation purposes.

These media were also sterilized (121 ± 1 °C and 1.1 atm) for 15 minutes in the autoclave (Prismatec 18L) and were then placed in Petri dishes of 9 cm in diameter (20 mL per plate) in a laminar flow chamber. Three plates were made for each treatment.

Statistics

The data were logarithmized to normalize the values and submitted to statistical analysis of variance through the Tukey and ANOVA test of a factor in the statistical program R (OKSANEN; KINDT; BLANCHET, 2013 - R core Team 2014). The data were represented through graphs with the averages of the triplicates of each treatment and control, together with the standard error of the samples in the Graphpad Prism Version 5.0 program.

Results and discussion

The different potassium sorbate concentrations tested on the percentage of mycelial inhibition, total growth (72 h), and daily growth rate (24 h) showed significant differences ($p < 0.001$) according to Table 1, evidencing the differences between the beginning of inoculation and up to 72 h of cultivation.

Table 1 – Data on the comparison of means (Tukey) in relation to the significance of mycelial growth inhibition, inhibition of growth after 72 h of incubation and every 24 h.

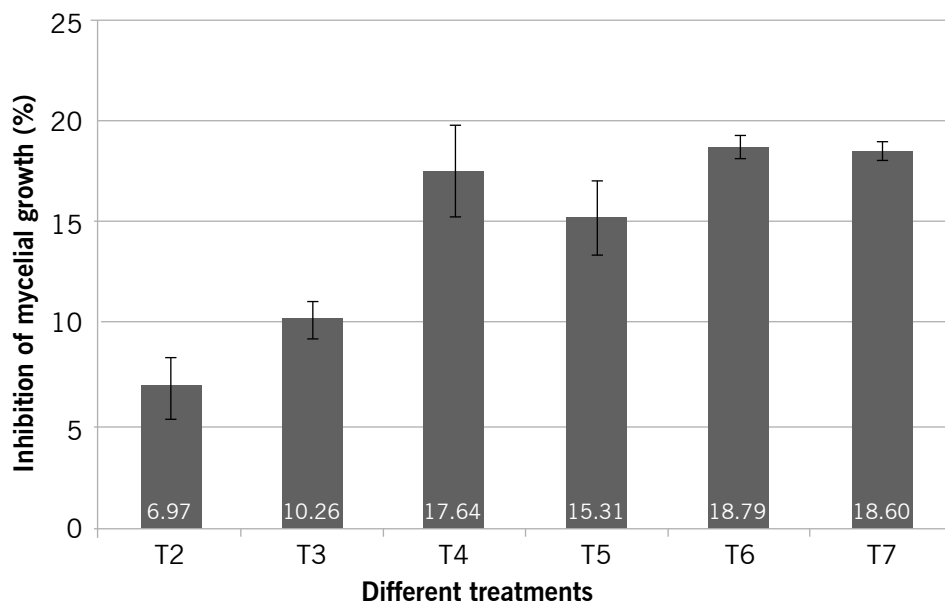
Variation	Treatments		
	d.f	F	P
Inhibition of mycelial growth	9	35.15	0,000000919
Growth after 72 hours of incubation	6	67.95	0,0000000151
Growth every 24 hours	6	67.95	0,0000000151

Source: Elaborated by the authors in University of São Paulo (2019).

Potassium sorbate is used to control different fungal species and their spores with effectively at some specific concentrations and their antifungal action has been attributed to enzyme inhibition, interference in substrate transport, and in the steps oxidative phosphorylation (FREESE; SHEU; GALLIERS, 1973; SOFOS, 1995). Its efficacy is also directly related to pH, and acid media cause greater inhibition in the development of fungi, bacteria, and yeasts (SOFOS, 1995).

In the Figure 1, it is possible to check that at the lowest concentrations (0.076 g L⁻¹ and 0.15 g L⁻¹) there was a lower inhibition of mycelial growth of the fungus *C. lindemuthianum* after 72 h of *in vitro* cultivation, and in the highest percentages of inhibition occurred in the highest concentrations, being 0.30 g L⁻¹, 0.45 g L⁻¹, 0.60 g L⁻¹, and 0.75 g L⁻¹ of the preservative potassium sorbate.

Figure 1 – Percentage of mean inhibition of mycelial growth of the fungus *C. lindemuthianum* after 72 h of *in vitro* cultivation under different concentrations of potassium sorbate.



Source: Elaborated by the authors in University of São Paulo (2019).

A few studies and/or reports inform that this preservative is effective in concentrations between 0.5 g L⁻¹ and 3.0 g L⁻¹ (BRASIL, 1999), not providing the percentage of inhibition or more information. Mecteau, Arul, Tweddell (2002) conducted a study analyzing the different effects of organic and inorganic salts against the phytopathogenic fungus *Fusarium sambucinum* causing rot in potato tubers. When analyzing mycelial growth and spore germination, the present study found inhibition

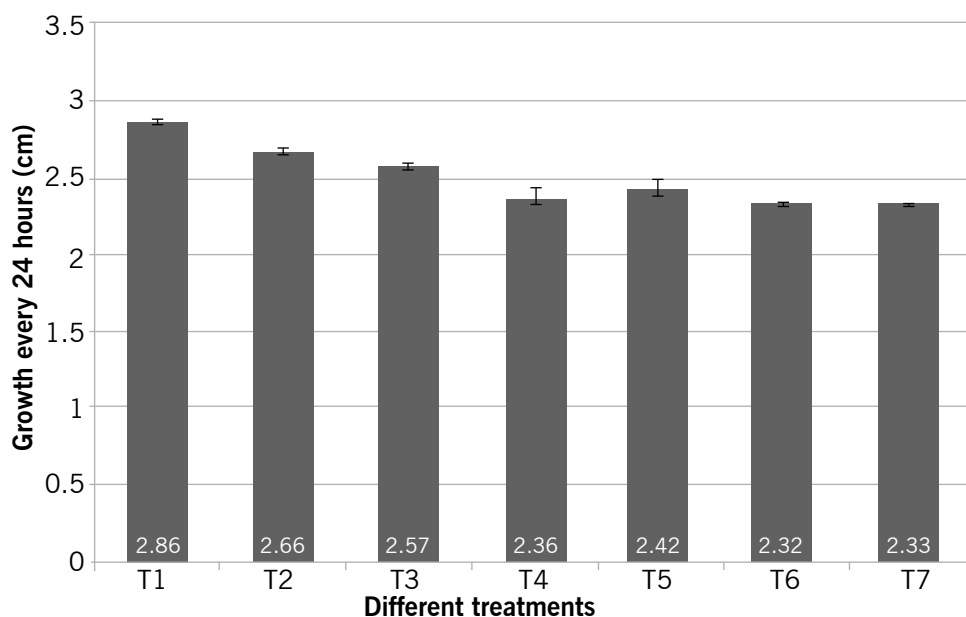
rates of 100 % for both conditions, when analyzing the concentration of 30 g L⁻¹ in 24 h of the experiment. In the present article, at the highest concentration (0.75 g L⁻¹) occurred an inhibition rate of 18.6 % and proportionally the inhibition rate was higher, considering that the concentration used was lower, as well as the exposure time was longer (72 h). Although it is also a phytopathogenic fungus, it is evidenced that the responses at the concentration level vary quite from one organism to another, and that high concentrations increase the effect as it is expected of a dose-dependent product.

Al Zaemey, Magan, and Thompson (1993) verified that potassium sorbate was efficient in completely suppressing the banana pathogen *Colletotrichum musae* at a concentration of 1.25 g L⁻¹ *in vitro* conditions. The highest concentration used in the present article was 0.75 g L⁻¹ and this was not efficient in completely suppressing the present phytopathogenic fungus belonging to the same genus, and this may have occurred due to the difference in concentration, which is about 1.6 times higher than used in the present article.

It was also found that at the concentration of 1,000 mg L⁻¹, it occurred 95 % inhibition of fungi that cause sinusitis when potassium sorbate was used *in vitro*. The highest concentration of the present study was 750 mg L⁻¹ and inhibition was much lower when compared with this study, considering, this time, no similarity to the genus of the tested species (ALSUDANI, 2017).

With the follow-up of mycelial growth every 24 h, a difference in the gain in centimeters was observed in the intervals of total growth, until the plaque was covered with mycelium of the fungus at the end of 72 h of cultivation. Lower growth averages were observed in the first two treatments with 0.076 g L⁻¹ and 0.15 g L⁻¹ (T2 and T3) with respect to control and growth averages even lower at concentrations of 0.30 g L⁻¹; 0,45 g L⁻¹; 0.60 g L⁻¹ and 0.75 g L⁻¹ (T4 to T7) of potassium sorbate per liter of BDA culture medium (FIGURE 2), thus evidencing that the higher concentrations of potassium sorbate promoted a lower average growth rate.

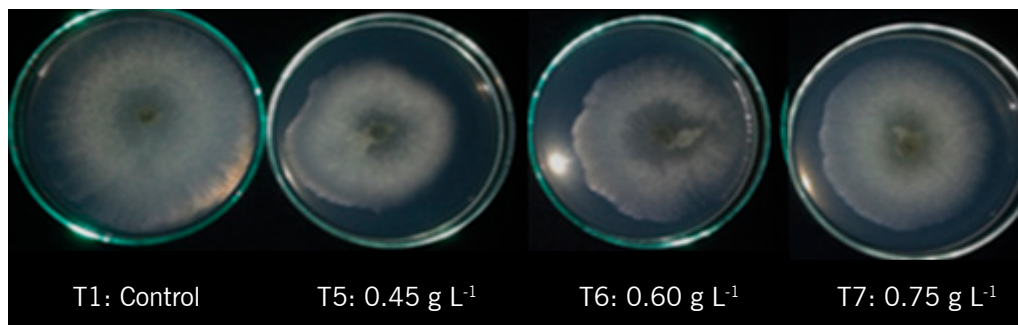
Figure 2 – Average growth in diameter (centimeters) of the fungus of *C. lindemuthianum* *in vitro* condition in the 24-hour interval under different potassium sorbate concentrations.



Source: Elaborated by the authors in University of São Paulo (2019).

It was also verified that after 72 h of *in vitro* cultivation the fungus *C. lindemuthianum* came to colonize 90 % of the plaque in T1 (control), and at the highest concentrations (T5 to T7: 0.45 g L⁻¹, 0.60 g L⁻¹, and 0.75 g L⁻¹) such colonization reached 70 %, causing, therefore, a reduction of plate colonization of 20 % (FIGURE 3).

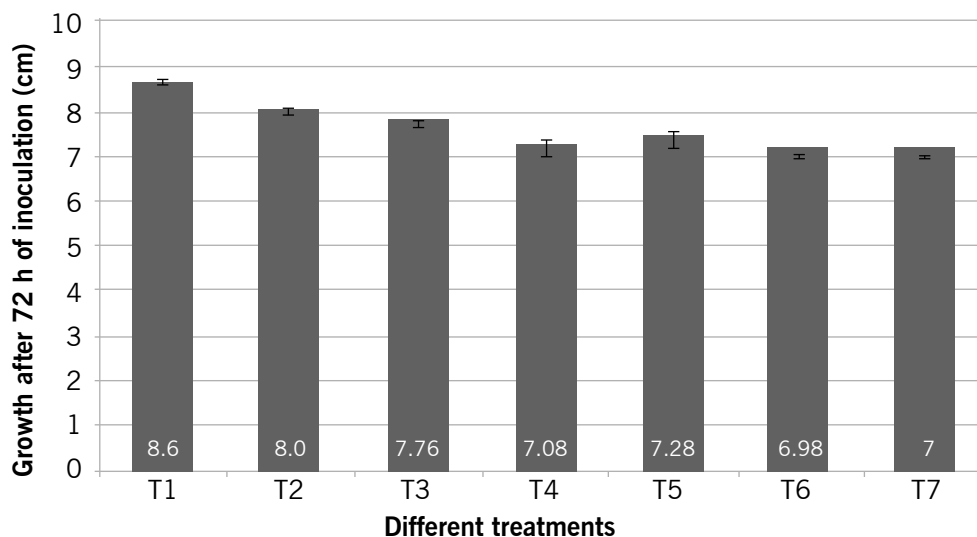
Figure 3 – Comparison of mycelial growth after 72 h of *in vitro* cultivation of T1, T5, T6, and T7 plates, respectively, evidencing the decrease in the colonization of the fungus *C. lindemuthianum* in the presence of potassium sorbate.



Source: Elaborated by the authors in University of São Paulo (2019).

These results were obtained from the average total growth of the present fungus after 72 h of cultivation (FIGURE 4).

Figure 4 – Comparison of the mean mycelial growth in diameter (centimeters) of the fungus *C. lindemuthianum* after 72 h of cultivation under different concentrations of potassium sorbate.



Source: Elaborated by the authors in University of São Paulo (2019).

It is noteworthy that although the plaque was colonized by the fungus even at the highest concentration of potassium sorbate (0.75 g L⁻¹), it is known that when manipulating an explant *in vitro* cultivation contamination occurs in much lower proportions than those practiced in the present study, considering all the asepsis processes that are previously performed according to studies (GRATTAPAGLIA; MACHADO, 1998; MORAES *et al.*, 2007). Thus, it is suggested that with the

asepsis performed previously and with the other care that should be adopted during the cultivate and the following phases of multiplication, the percentage of inhibition can certainly be well above the 20 % observed, it and may even result in a material free of contamination.

It is already known that there are several alternatives in the control of fungal contamination *in vitro* cultivation, such as those mentioned previously and in relation to their use in the culture media, studies have revealed that the use of some fungicides such as Benomil, Chlorothalonil, and Azoxistrobin demonstrated lethal effects for explants of a orchid and eucalyptus specie, even at the lowest concentrations studied, also evidencing its toxic characteristics to the environment (WATT; GAUNTLETT; BLAKEWAY, 1996; ODA *et al.*, 2003), and therefore, potassium sorbate may add up as a strategy to combat fungi observed *in vitro*, by the characteristics already presented.

Thus, considering that there are still some gaps in the disinfection processes for *in vitro* multiplication, as well as risks and unfeasibility in using commercial fungicides to combat fungal contamination *in vitro*, the food preservative potassium sorbate can be a product to be used to control the development of fungi in plant crops in the *in vitro* multiplication stage, given the results presented, which may help in a significant reduction of plant material losses due to contamination, which, in some cases, may cause the unsuccess of *in vitro* multiplication of plant species with consequent commercial loss, as well as the infeasibility of these *in vitro* cell culture laboratories (CASSELS, 1991; CASSELLS, 1997).

Conclusions

Treatments from T4 to T7 had statistically equal and higher inhibition performances in relation to T2 and T3 treatments. The highest inhibition percentages occurred from T5 to T7 with an average inhibition rate of 17.5 % for the 72 h period, although the increase in potassium sorbate concentration also caused a higher percentage of inhibition, regardless of time, in all treatments.

The present study indicated that potassium sorbate can provide a control of the fungus *C. lindemuthianum*, and more information about its antifungal activities in the combat of *in vitro* microorganisms correlated with plant cell cultivation need to be elucidated.

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Inibição do fungo *Colletotrichum lindemuthianum* na presença de diferentes concentrações de sorbato de potássio

Resumo

Um dos grandes problemas do cultivo vegetal *in vitro* é causado por contaminações como as fúngicas, que representam grandes perdas para os laboratórios de cultivo vegetal. Diante da problemática acerca das contaminações e das lacunas em protocolos eficientes de desinfecção, o presente estudo teve como objetivo avaliar a inibição do fungo *Colletotrichum lindemuthianum* na presença do conservante alimentar Sorbato de Potássio em diferentes concentrações (T1: controle, T2: 0,076 g L⁻¹;

T3: 0,15 g L⁻¹; T4: 0,30 g L⁻¹; T5: 0,45 g L⁻¹; T6: 0,60 g L⁻¹ e T7: 0,75 g L⁻¹). Quantificaram-se as análises da Porcentagem de Inibição do Crescimento Micelial (PIC) e o crescimento em 24 e 72 horas em diferentes concentrações. Observou-se que o Sorbato de Potássio foi efetivo na análise de PIC nas diferentes concentrações com relação ao controle, com maior efetividade para o T4 a T7. As maiores porcentagens de inibição (T5 a T7) tiveram uma taxa de inibição média de 17,5 % em relação ao controle para o período de 72 h, indicando, portanto, que com mais estudos este conservante pode ser utilizado no combate a microrganismos fúngicos no cultivo *in vitro*.

Palavras-chave: Inibição de microrganismos. Contaminação *in vitro*. Inibição fúngica. Crescimento micelial. Conservante alimentar.

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