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Effects of plant extracts and sodium hypochlorite on lettuce germination and inhibition of *Cercospora longissima in vitro*

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ABSTRACT

Vegetables that are propagated by seeds are considered to be commercially and nutritionally valuable. However, there is a need to overcome the present challenge of developing alternative technologies for organic horticulture. This paper evaluates the potential use of the aqueous extracts of clove, cinnamon, and coriander and the sodium hypochlorite in the germination of lettuce (*Lactuca sativa*) seeds and in the control of *Cercospora* disease. Two experimental groups were thus designed: 1) the effects of the different aqueous extracts on germination and initial development of lettuce seeds were observed and; 2) the effects of these extracts on the mycelial growth of the fungus *Cercospora longissima* (isolated from lettuce plants) were evaluated. The treatments used in the first group were control (with water only), 5% (v/v) sodium hypochlorite (NaOCl) and 5% (w/v) aqueous extracts of clove, cinnamon, and coriander. The pretreatment of seeds was performed just before placing them for germination by immersing in these solutions. In the second set of the experiment, the inhibition of mycelial growth of the *Cercospora longissima*, isolated from lettuce plants presenting cercosporiosis symptoms, was evaluated. In the seeds treated with clove and cinnamon extracts, the germination speed index was negatively affected, while those treated with coriander extract showed an increase in the fresh mass of the aerial part and in the number of leaves of lettuce seedlings. In the second experimental group, it was observed that the growth of *Cercospora longissima* was inhibited completely by the cinnamon extract and by sodium hypochlorite.

1. Introduction

Most of the cultivated plant species used for food are propagated by seeds. The health of such seeds is of utmost importance as there might be possibility of the survival and transmission of phytopathogens. The pathogens are present as inocula within or on the surface of the seeds and sometimes even in the seed lot, thereby introducing the diseases into previously clean areas (Henning, 2005). According to Souza et al. (2007), fungi are the biggest group of pathogenic agents associated with seeds and grains.

The usage of certain products in seed treatment has been found to effectively reduce or annul mycelial growth on the surface of grains and seeds (Souza et al., 2007). A great part of the seed commercialization involves treatment of the contact fungicides (Strandberg, 1984; Kaewkham et al., 2016), which are banned in some horticulture production systems. Therefore, it is necessary to develop alternative methods, like physical treatment, microbial treatment and treatment with natural agents like plant extracts, which are capable of preventing or controlling the microorganisms in the seeds (Koch and Roberts, 2014).

According to Venturoso et al. (2011), herbal byproducts, in the form of crude extracts and essential oils, are alternatives to the synthetic products. These extracts contain microbiocide active substances that give them a potential to be used against phytopathogens in the plant defense. Hong et al., (2015) suggested that the potent fungicidal nature of the plant extracts and essential oils makes them useful in the control of various phytopathogens.

It is important, therefore, to emphasize, that high-quality seeds promote rapid germination and give rise to normal and healthy seedlings. The seedling, which are free from contamination and have essential structures developed, are extremely important for horticulture (Minami, 2003; Mancini and Romanazzi, 2014; Lima et al., 2016; Kaewkham et al., 2016), especially in organic farming.

In addition, the use of natural substances replaces and diminishes the potential toxic effect of pesticides on human beings (Pereira et al., 2006). The investigations that have employed essential oils and plant extracts for the treatment of seeds of vegetable crops have reported a significant reduction in the seed pathogens (Lima et al., 2016).

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However, it is important to point out that some of the herbal compounds may hinder the development and germination of other plants (Mairesee et al., 2007). In this context lettuce (*Lactuca sativa*) is one of the most important vegetables of the world to be propagated by seeds and its leaves are mostly consumed fresh. Lettuce is used as a bioindicator model in different allelopathy tests (Lima et al., 2016).

Among the plant species, clove (*Syzygium aromaticum*) and cinnamon (*Cinnamomum* sp.) have a high potential to control pathogens. Besides clove, eugenol is also found as one of the constituents of essential oil of cinnamon, whose fungicide potential is reported in the literature (Rao and Gan, 2014; Wang et al., 2010). According to Mandal and Mandal (2015), coriander contains linalool, a substance with antifungal and antioxidant potential.

The aim of this paper is to evaluate the possible effects of plant extracts and of sodium hypochlorite in the treatment of lettuce seeds and in the inhibition of mycelial growth of the *Cercospora longissima* fungus, which was isolated *in vitro* from diseased lettuce plants.

2. Materials and methods

2.1. Allelopathic effects of aqueous extracts and of sodium hypochlorite on lettuce seed germination

The lettuce cv. 'Hanson' (Feltrin™, Farroupilha, Brazil) type curled cabbage, lettuce seeds were used for the germination tests.

Five treatments were given, namely, aqueous extract of cinnamon (*Cinnamomum verum*), aqueous extract of coriander (*Coriandrum sativum*), aqueous extract of clove (*Syzygium aromaticum*), sodium hypochlorite (2.0–2.5% active chlorine), and deionized water (control).

Plant materials were obtained from commercial products (Siamar®, Neves Paulista, Brazil) stored at a temperature of 20–28 °C until the time of preparation. The aqueous extracts of coriander, clove, and cinnamon were prepared at the concentration of 5% (*w/v*) by grinding 5 g of the dry mass of coriander leaves (powder), floral clove buttons (powder), and cinnamon bark (flakes), respectively, in 250 mL Erlenmeyer flasks containing 100 mL of deionized water. The preparation of extracts was carried out by heating the solution for 5 min at 100 °C in a bain-marie. For sodium hypochlorite treatment, a commercial solution of bleach (Candura®, Piracicaba, Brazil) (2.0–2.5% of active chlorine) was used at the final concentration of 5% (*v/v*) (0.10–0.125% active chlorine).

The lettuce seeds were left immersed in the treatments for 30 min after which they were counted and placed in 100 × 15 mm Petri dishes containing filter paper moistened with deionized water. Ten seeds were placed in each Petri dish and the experiment was conducted as completely randomized block design that contained four repetitions (Petri dishes) each of all the five treatments. The Petri dishes containing the seeds were kept randomly in a growth room and maintained at 25 ± 2 °C, with light source supplied by white fluorescent lamps (photoperiod of 14 h and 2500 lx of luminosity for 15 days). The experiment was repeated twice and the means were obtained from the repetitions of the two experiment replicates, totalizing five treatments with eight repetitions each.

The germination count was performed daily for 15 consecutive days and the Germination Speed Index (GSI) was calculated according to the formula proposed by Maguire (1962):

GSI = E1/N1 + E2/N2 + ... En/Nn

- GSI = germination speed index;
- E1, E2… En = number of normal plants computed at the first count, second count and so on until the last count;
- N1, N2… Nn = number of days of sowing the first count, the second count, and so on until the last count.

The total germination percentage data were collected on the 15th day and the seeds, emitting radicle and resuming the growth of the embryo, were considered to have germinated. Additionally, the total fresh mass of the aerial part and root, the root length and the number of leaves of lettuce were determined. The mass values were measured using a digital weighing scale (Mettler Toledo, ML204) with the precision up to four decimal places and the plant length was measured with the aid of a caliper.

The resulting data were statistically tested by analysis of variance (ANOVA) and the means were compared using Duncan's test at 1% or 5% probability. All the tests were performed with the aid of Assistat Software.

2.2. The in vitro growth of Cercospora longissima in different aqueous extracts and in sodium hypochlorite solution

The *Cercospora* was isolated by collecting lettuce plants that displayed disease symptoms. The collected material was rinsed with running water and fragmented, following which sterilization was performed (in the laminar flow cabinet) by the immersion of the material in 70% alcohol for ten seconds and in the sodium hypochlorite (2.0–2.5% of active chlorine) solution for five min. The fragments were then rinsed twice with sterile distilled water and placed in Petri dishes containing potato dextrose agar (PDA) and grown at 25 °C. The PDA was prepared with 200 g of potato (potato broth), 10 g of agar, and 18 g of dextrose in 1 L of distilled water.

After seven days of incubation period, the colony purification was done by transferring the fragments of PDA from the border of the better-developed colonies, onto fresh Petri dishes with PDA and growing them at 25 ± 2 °C. The entire process was repeated three times. Later the fungus was identified by observing the conidia under a light microscope and the identity was confirmed by the laboratory of molecular genetics (CCA/UFSCar).

The experimental design was completely randomized blocks with five treatments and five repetitions per treatment. The tests employed PDA with aqueous extract of cinnamon (*Cinnamomum verum*), or coriander (*Coriandrum sativum*), or clove (*Syzygium aromaticum*), or PDA with sodium hypochlorite, and PDA with the control. The stock solutions of cinnamon, clove, and coriander aqueous extracts were prepared at 25% concentration, by keeping in a bain-marie for 5 min after the boiling point. The hypochlorite was used at the concentration of 25% (2.0–2.5% of active chlorine).

To test the inhibition of fungal mycelial growth, the extracts and hypochlorite were mixed with PDA growth medium to make up to a final concentration of 5% (*v/v*) (inside a laminar flow chamber) and plated on the Petri dishes. In case of control, only the PDA growth medium was plated on the Petri dishes. The solutions were added to the PDA growth medium after cooling down to 40–60 °C to avoid decomposition of the active products present in the extracts.

After solidification of the medium, PDA fragments of approximately 0.5 cm containing purified mycelia of the *Cercospora longissima* fungus, collected from the borders of the colonies, were transferred to the center of a fresh Petri dish. The Petri dishes were incubated in a growth room at 25 ± 2 °C.

Mycelial growth was evaluated daily for 15 days by measuring the colony diameter (in cm) and plotting the mycelial growth curve. The Venturoso et al. (2011) formula, PIG = [(diameter of the control – diameter of the treatment) / diameter of the control] × 100, was used to obtain the percentage inhibition of growth (PIG) of phytopathogen when exposed to an extract in comparison with the control. The analysis of variance (ANOVA) was applied to the resulting data using Assistat Software and the averages were compared with Duncan's test at 1% probability. The phytopathogen growth rate was obtained through the simple linear regression equation; (y = a + bx), where (x) is days of incubation, (y) is colony's final diameter, (a) is initial diameter, and (b) is the mycelial growth rate, obtained from the regression coefficient (Venturoso et al., 2009). The calculation of the IVCM was