Use of water restriction in the inoculation of carrot seeds[1]

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ABSTRACT – This study aimed at evaluating the method of inoculation of carrot seeds in a potato-dextrose-agar medium (PDA) added of mannitol. *Alternaria radicina* was the fungus chosen to test such methodology. The osmosis-controlling agent was employed to obtain the osmotic potentials of 0.6 MPa, 0.8 MPa, 1.0 MPa and 1.2 MPa. In comparison with the control treatment, no significant mycelial growth inhibition was noticed at any of the osmotic potentials considered. The seeds were distributed over the fungal colonies for infection, where they remained for 48, 72, 96, 120 and 144 hours, at the osmotic potentials of 0.0 MPa (control), 0.6 MPa, 0.8 MPa, 1.0 MPa and 1.2 MPa, respectively. Tests of germination, sanitization and emergence were performed to evaluate the effects of colonization. Regarding the germination, the different osmotic potentials resulted in similar percentages of abnormal seedlings and dead seeds. As for the sanitization testing, higher infection rates were observed at the osmotic potentials of 1.0 MPa and 1.2 MPa. The lowest percentage of emergence was found at -1.2 MPa, therefore suggesting that this osmotic potential (produced with mannitol) might be used for infecting carrot seeds for research purposes.

Index terms: *Daucus carota,* mannitol, seeds infection.

Uso de restrição hídrica na inoculação de sementes de cenoura

RESUMO – O objetivo deste trabalho foi avaliar o método de inoculação de sementes de cenoura com meio batata-dextrose-ágar (BDA) acrescido de manitol. O fungo escolhido para testar a metodologia foi o *Alternaria radicina.* O restritor hídrico foi adicionado ao meio BDA de modo a obter os potenciais osmóticos de 0,6 MPa, 0,8 MPa, 1,0 MPA e 1,2 MPa. O crescimento micelial foi avaliado, e não foi observado inibição do crescimento em nenhum dos potenciais osmóticos, em relação a testemunha. As sementes foram distribuídas sobre as colônias fúngicas para a infecção, onde permaneceram por 48, 72, 96, 120 e 144 horas nos potenciais osmóticos 0,0 MPa (controle), 0,6 MPa, 0,8 MPa, 1,0 MPa e 1,2 MPa, respectivamente. Para avaliar os efeitos da colonização foram realizados testes de germinação, sanidade e emergência. Os potenciais osmóticos avaliados apresentaram porcentagens semelhantes de plântulas anormais e sementes mortas no teste de germinação. No teste de sanidade, maiores porcentagens de infecção foram observadas nos potenciais osmóticos 1,0 MPa e 1,2 MPa. O potencial -1,2 MPa apresentou a menor porcentagem de emergência. Assim, o uso de manitol -1,2 MPa é sugerido como um método eficaz para a infecção de sementes de cenoura com para fins de pesquisa.

Termos para indexação: *Daucus carota*, manitol, infecção de sementes.

Introduction

Research on the control of seed-borne pathogens often depends on the availability of contaminated grains. For that reason, it is necessary to develop efficient techniques that result in high seed infection rates. Traditionally, seeds are exposed to fungi via imbibition in a suspension of conidia (Pedroso et al., 2010), or through contact with fungal structures (mycelia) grown in conventional culture media. Nonetheless, according to Machado et al. (2001), such practices do not guarantee seed inoculation, once the pathogen might just adhere to the integument, not reaching the inner tissues of the seed.

For that reason, the method of water restriction induced by mannitol ($C_6H_{14}O_6$) is an interesting alternative, as it

inhibits germination for more extended incubation periods, giving it time for the infection process to take place (Costa et al., 2003; Carvalho et al., 2004). According to Ávila et al. (2007), mannitol has frequently been employed as an osmotic agent to simulate water deficits and, since it is chemically inert, it does not cause structural harm to seeds. However, such premise is not entirely valid, since Menezes et al. (2011) reported the influence of this compound, at different osmotic potentials, on the physiological quality of cucumber seeds.

Some works have already proved the efficiency of this osmosis-controlling agent in facilitating pathogen inoculation in seeds. Machado et al. (2004), for example, used mannitol in a PDA medium (200 g of potato extract, 20 g of dextrose, and 20 g of agar), in order to adjust the osmotic potential to values between 0.8 MPa and 1.0 MPa. On that occasion, they obtained good indices of cotton seed infection with *Colletotrichum gossypii* South var. *cephalosporioides* Costa, *Botryodiplodia theobromae* (Pat.) Griffon and Maubl., and *Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) Snyder and Hansennos. Machado et al. (2001), using the same methodology but at 0.8 MPa to 1.2 MPa, attained high infection rates of maize seeds with the fungi *Diplodia maydis* (Berk.) Sacc., *Cephalosporium acremonium* Corda and *Fusarium moniliforme* J. Sheldon.

In vegetables, the use of water restriction to infect seeds remains little investigated. In works conducted by the phytopathology study group of *Embrapa Hortaliças* [Brazilian Agricultural Research Corporation], the method of mannitol in a PDA medium proved to be efficient in aiding the inoculation of onion seeds with *Colletotrichum gloeosporioides* f. sp. *cepae* (Penz.) Penz. and Sacc. They got the best infection percentages at the osmotic potentials of 1.0 MPa and 1.2 MPa.

Infected carrot seeds for epidemiologic studies and other purposes are difficult to find. On that account, this work aimed at evaluating the use of mannitol in culture media at different water potentials, in order to achieve high infection rates of carrot seeds by the fungus *Alternaria radicina*.

Material and Methods

The research was carried out from November 2015 to February 2016, in the Laboratory of Phytopathology and Laboratory of Seeds, as well as in a greenhouse, all of them belonging to *Embrapa Hortaliças*, located in *Brasília, Distrito Federal*.

Carrot leaves exhibiting blight symptoms were collected from an experimental field and then taken to the laboratory, where it was subjected to monosporic isolation of *A. radicina*.

Next, the fungus was grown in a PDA medium (200 g of potato extract, 20 g of dextrose, 20 g of agar) and identified. All assays utilized carrot seeds (cv. Kuronan) from a single lot. They had their vigor and emergence appraised and were deemed as adequate for performing the experiments (at 94% emergence).

Effect of water restriction on A. radicina development

To assess the effect of water restriction upon *A. radicina* development, mycelia of the pathogen were cultivated in a PDA medium added of mannitol (182.172 g/mol), so as to induce water restriction. The osmotic potentials of 0.0 MPa (control treatment, mannitol-free PDA media), -0.6 MPa, -0.8 MPa, -1.0 MPa and -1.2 MPa were considered.

The concentrations used to prepare the culture media of each water potential were determined by the Van't Hoff equation (Souza and Cardoso, 2000):

$$Po = C \times R \times T$$

Where Po = water potential (MPa); R = universal gas constant (0.00831 kg MPa/mol/K); T = absolute temperature (°C + 273); and C = concentration (moles/kg of water). The temperature of 25 °C was assumed as reference in this case.

After the media containing mannitol had been prepared, approximately 25 mL was poured into Petri dishes. Once they solidified, a 5-millimeter disc carrying culture medium with young mycelia of the fungus was placed in the center of each unit. Next, the dishes were sealed with transparent plastic film and stowed inside a BOD incubator chamber set at 25 ºC and 12-hour photoperiod.

The experiment complied with a completely randomized design, with five treatments and seven replications, each one composed of a 90-millimeter Petri dish. The diameter of the colonies was measured periodically, for seven days, until the fungus colony in the control treatment covered the whole media surface. The mycelial growth rate (MGR) was determined through the equation proposed by Maguire (1962).

Seed inoculation with A. radicina

Carrot seeds were previously disinfected in a 1% sodium hypochlorite solution for 1 min. Then, they were rinsed with distilled water and left to dry at room temperature for about 1 h.

The fungus totally overspread the culture media eight days after it had been streaked in all osmotic potential treatments. So, 160 seeds were placed on each dish, gently pressing them over the mycelia with a Drigalski spatula. After that, the sets returned to the incubator (at 25 °C and a 12-hour photoperiod), where they remained until at least one seed within each treatment started to show radicle protrusion. That way, seeds were taken off of the incubator after 48, 72, 96, 120 and 144

hours, corresponding to the osmotic potential of 0.0 MPa, -0.6 MPa, -0.8 MPa, -1.0 MPa and -1.2 MPa, respectively.

The seeds were laid over filter paper to dry at room temperature for about 1 h. The result was small lots, each one containing around 1,400 seeds from each treatment. Subsequently, these sets were tested for germination, sanitization effect and emergence in soil, in order to appraise the efficacy of the infection method.

Germination test

The seeds were distributed in plastic Gerboxes containing two germination-paper sheets, which had been previously moistened with distilled water at a ratio of 2.5 times the dry substrate weight. After the boxes had been set up with fifty seeds in each, they were placed inside a germinator adjusted to 25 ºC and 12-hour photoperiod, where they stayed for seven days before the testing. Besides the treatments holding infected seeds and the osmosis-controlling agent, another one was included, containing uninoculated seeds (absolute control).

The experiment was performed in a completely randomized design with six treatments and four replications, each one represented by a Gerbox enclosing fifty seeds. The analyses determined the percentages of normal and abnormal seedlings, as well as the rate of dead seeds, according to the Rules for Seed Testing (Brasil, 2009). The normal seedlings comprised those exhibiting intact vital structures, those with less than 50% malformed tissues, and also the ones with infection signs (as long as the seed was not the source of it). In turn, seedlings with either damaged or poorly formed essential structures, or which showed fungal deterioration or albinism traces were deemed abnormal. Lastly, non-germinated seeds, which by the end of the test presented themselves swollen and infected by the pathogen, were considered dead.

Sanitization testing

The sanitization test was evaluated via incubation in a paper substrate. This analysis contemplated not only the experimental groups combining infected seeds and the osmosis-regulating agent, but also an additional one, with uninoculated seeds (absolute control). One hundred grains from each of those treatments were sanitized separately in a 1.0% sodium hypochlorite solution for 1 min; while another similar set of one hundred seeds remained disinfected. Twenty-five seeds from each treatment were spread inside plastic Gerboxes containing two germination-paper sheets, which had been previously moistened with distilled water at the ratio of 2.5 times the dry substrate weight. Next, the boxes were placed inside an incubation chamber set at 25 ºC, for the first 24 h, then at -20 ºC, for another 24 h and,

finally, at 25 ºC and 12-hour photoperiod, for seven days prior to the evaluation.

The experiment employed a completely randomized design, considering twelve treatments (with and without sodium hypochlorite) and four replications, each one representing one Gerbox with 25 seeds. The effect of sanitization was checked by observing the seeds in a stereomicroscope. The seeds that had been disinfected with sodium hypochlorite and later presented *A. radicina* reproductive structures were considered contaminated.

Emergence test

Four subsamples with one hundred seeds from each treatment – the ones containing osmosis-controlling agents and also that with uninoculated seeds (absolute control) – were sown at 1.5 centimeter depth, in plastic trays filled with autoclaved substrate. This substrate had been prepared by mixing 85% of soil, 5% of dried rice husk and 10% of carbonized rice husk (v:v). After sowing, the sets were randomly distributed inside a greenhouse, where they were irrigated as needed, with equal volumes of water to each tray.

This experiment followed a completely randomized design, with six treatments and four replications, each one consisting of a tray with fifty seeds. The assessments were performed seven and 21 days after sowing. In the last evaluation, the seedlings were collected and analyzed for symptoms of disease caused by *A. radicina*. The results were conveyed in percentage of normal seedlings.

Statistical analysis

The data from the tests of germination, sanitization and emergence were subjected to variance analysis, and the means were grouped by the Scott-Knott test ($p \leq 0.05$). All statistical procedures were handled by the software Sisvar (v.5.6).

Results and Discussion

Effect of water restriction on A. radicina growth

The osmotic potentials of 0.6 MPa to 1.2 MPa, promoted by the addition of mannitol to the culture media, did not significantly affect *A. radicina* mycelial development. Similar results were observed by Carvalho et al. (2009), who assessed the proliferation of *A. dauci* in PDA media adjusted with mannitol to between 0.4 MPa and 1.2 MPa. On the other hand, the same authors confirmed a substantial decrease in *A. radicina* growth at the values of 1.0 MPa and 1.2 MPa. Such a difference could be attributed to characteristics of either the isolate or cultivation conditions, like temperature and photoperiod. More recently, Reis et al. (2014) verified that

the addition of mannitol to PDA media, so as to achieve the osmotic potentials of 0.3 MPa, 0.6 MPa and 0.9 MPa, did not curb the mycelial development of *Sclerotinia sclerotiorum*.

Germination test

The test showed that seeds that had not been exposed to contamination (absolute control) had the highest germination rates (89%). The following highest values were achieved by the control treatment (PDA medium with no mannitol) and those with osmotic potentials of -0.6 MPa and 0.8 MPa - in fact, these sets did not statistically differ from one another, exhibiting from 6% to 11% of normal seedling emergence (Figure 1). The seeds inoculated at 1.0 MPa and 1.2 MPa presented the lowest percentages, with 0.5% and 1.0% of emerged normal seedlings, respectively.

Except for the absolute control, which scored 0.5% of abnormal seedlings and 14% of dead seeds, the remaining experimental groups with contaminated seed presented superior rates of abnormal seedlings (from 57% to 71%) and dead seeds (from 29% to 42%), regardless of the osmotic potential considered. Also, they did not differ from one another nor from the PDA control treatment.

According to the observed results, the inoculated treatments prompted high percentages of both abnormal seedlings and perished seeds (95%, on average). However, significant augments in the number of dead seeds might make the infection unfeasible for any further use, including epidemiologic studies and the development of new methods

for managing and treating seeds.

In the present study, the ratio of abnormal seedlings and dead seeds was somewhat similar among the inoculated experimental groups, even though the more prolonged exposition of seeds matched the higher osmotic potentials. Machado et al. (2001) used similar potential values and verified that maize seeds contaminated with the fungi *Diplodia maydis*, *Cephalosporium acremonium* and *Fusarium moniliforme* displayed higher percentages of abnormal seedlings at the most negative osmotic potentials. Costa et al. (2003), when employing different solutes and exposure times, realized that the longer the contact with *Fusarium oxysporum* f. sp. *phaseoli*, the more compromised the germination of bean seeds. Ultimately, Machado et al. (2004) proved that cotton seeds inoculated with *Colletotrichum gossypii*, *C. gossypii* var. *cephalosporioides* and *F. oxysporum* f. sp. *Vasinfectum* also presented percentages of abnormal seedlings and unviable seeds significantly higher at the negative osmotic potentials, in comparison with the control treatments.

According to the authors, the expressive rise in the number of abnormal seedlings and dead seeds is a reflex of the higher inoculation potential, as a consequence of the more extended contact with the pathogen. Nevertheless, seeds exposed to high osmotic potentials for too long may lose their germination capacity, as Teixeira et al. (2005) detected in maize seeds subjected to osmotic potentials above 1.0 MPa for eight incubation days. Such results indicate that the physiological quality of the seeds might be affected by both
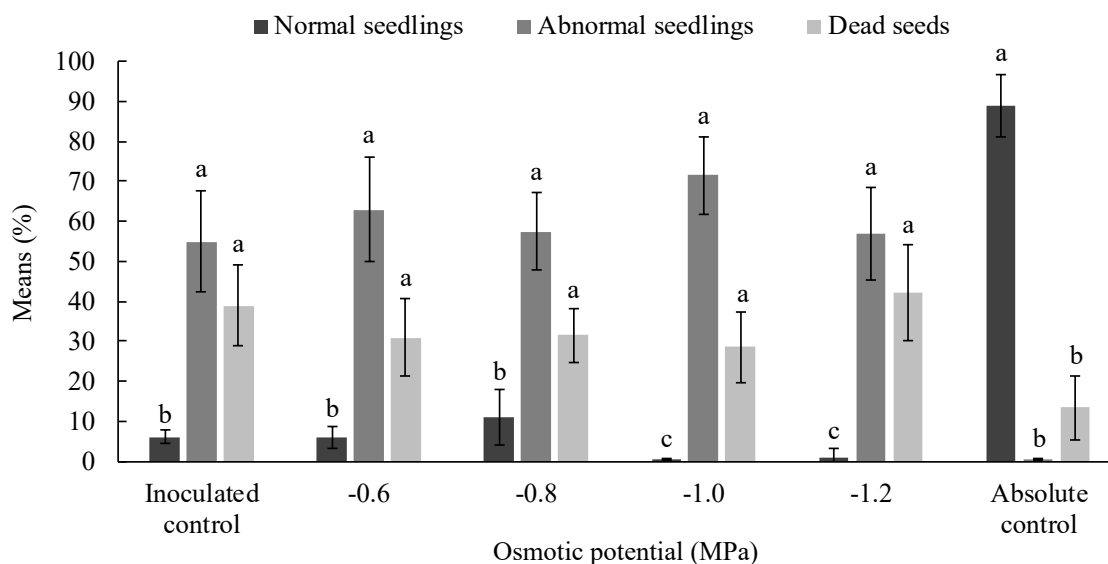

Figure 1. Percentage of normal and abnormal emerged seedlings and dead carrot seeds seven days after inoculation with *A. radicina* at different osmotic potentials.

Means followed by the same letter statistically do not differ, according to Tukey's test at a 5% probability level.

the exposure time and the osmotic conditioning imposed by the culture medium.

Sanitization testing

In the Sanitization testing, 100% of the non-disinfected seeds got infested after exposition to *A. radicina* in a PDA medium, either pure (control treatment) or in combination with mannitol (Figure 2). On the other hand, the seeds that had been contaminated and then sanitized with a sodium hypochlorite solution showed significant reductions in the infestation rates at the osmotic potentials of 0.6 MPa (60%) and 0.8 MPa (65%) – in these cases, the results did not differ from the control treatment. The seeds subjected to 1.0 MPa and 1.2 MPa also presented 100% infestation. Ultimately, in the absolute control, the pathogen was detected in neither infected nor disinfected seeds.

Galli et al. (2005) defined infested seeds as those in which the pathogen is adhered to the grain surface, whereas the infected ones have it in their inner tissues. Once the disinfection with sodium hypochlorite had removed the *A. radicina* inoculum from seed pericarp, the osmotic potentials of 1.0 MPa and 1.2 MPa proved to be the most efficient configurations to promote the contamination of the seeds. This outcome is probably related to the more extended exposition to the fungus (120 h and 144 h, respectively). In the mannitol-free PDA medium and in those with low water restriction (0.6 MPa and 0.8MPa), there was an enormous and significant difference between the infection rates of disinfested and non-disinfested seeds. In these cases, the seeds had remained less time in contact with the fungus (48, 72 and

96 h, respectively), which suggests that the exposure was not enough for the pathogen to penetrate to the inner structures.

Costa et al. (2003) observed that prolonged periods of incubation of bean seeds led to higher percentages of colonization by *F. oxysporum* f. sp. *phaseoli*, consequently producing different levels of infestation. In turn, Machado et al. (2001) concluded that maize seeds inoculated with *D. maydis* and *C. acremonium* conveyed higher infection rates when kept at a more negative osmotic potential (1.2 MPa for 120 h). Similar results were achieved by Barrocas et al. (2014) when researching cotton seeds treated with *C. gossypii* var. *cephalosporioides*. Conversely, this very study revealed a high percentage of infection in maize seeds inoculated with the fungus *F. moniliforme*, at the osmotic potentials of 0.4 MPa to 1.0 MPa and also without water restriction. This is an indication that this pathogen grows fast and easily penetrates the protective layers of the seed. Ultimately, Machado et al. (2004) noticed considerable differences in the infection rates of cotton seeds that had been contaminated with *C. gossypii*, *C. gossypii* var. *cephalosporioides*, *Botryodiplodia theobromae* and *F. oxysporum* f. sp. *vasinfectum* at 0.4 MPa to 1.2 MPa.

All facts considered, it is safe to say that the efficiency of the infection method varies enormously, depending on the osmotic potential, the seed species and the pathogen used in each case.

Test of emergence in soil

Carrot seeds that had been subjected to inoculation without water restriction showed emergence of 22% after 7 DAS. For the same period, seeds that had been treated at 0.6 MPa exhibited 11% emergence (Figure 3), whereas those exposed to more negative osmotic potentials or that had not
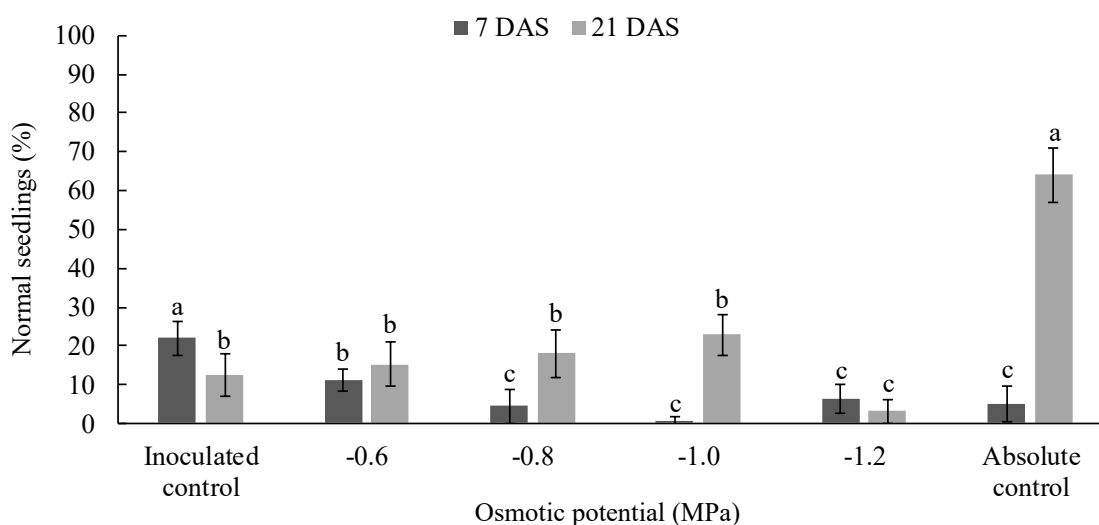

Figure 2. Percentage of infestation by *Alternaria radicina* of carrot seeds seven days after inoculation at different osmotic potentials, disinfested and non-disinfested with sodium hypochlorite solution.
Means followed by the same letter statistically do not differ, according to Tukey's test at a 5% probability level.
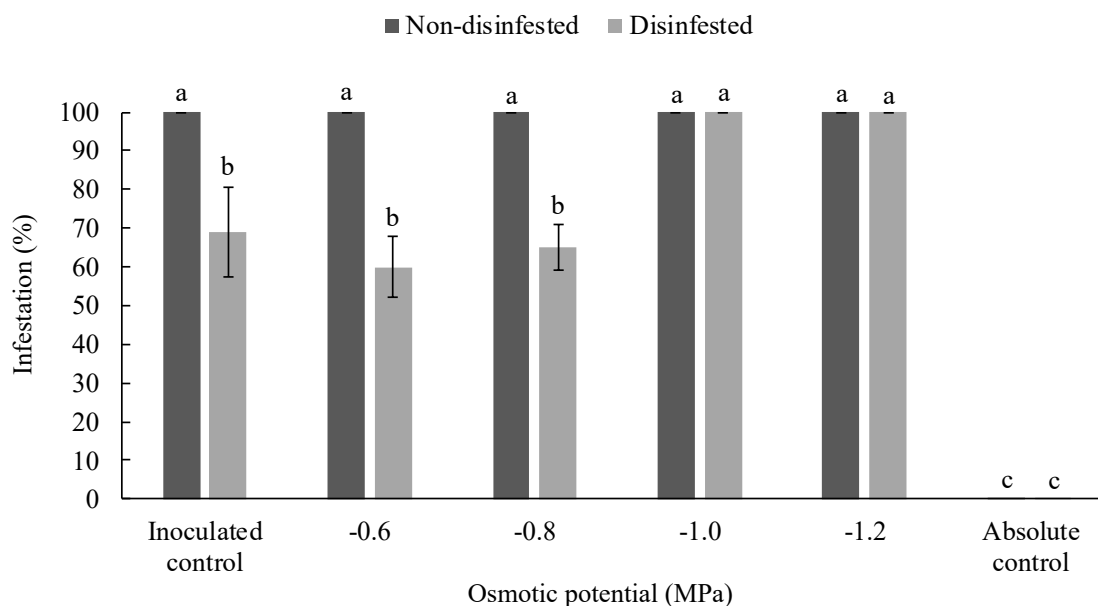
Figure 3. Percentage of normal carrot seedlings seven and 21 days after sowing (DAS). The seeds were inoculated with *A. radicina* at different osmotic potentials.
Means followed by the same letter statistically do not differ, according to Tukey's test at a 5% probability level.

been inoculated revealed a similar emergence behavior, with values below 6%. By 21 DAS, the non-inoculated seeds scored 64% of emergence, followed by the seeds tested at 0.6 MPa, 0.8 MPa, 1.0 MPa and the control treatment – these groups did not differ among themselves, with emergence ranging from 12% to 23%. For the same span, seeds inoculated at -1.2 MPa had the lowest emergence (3%).

Non-inoculated seeds showed higher emergence by the 7th DAS, probably because they were maintained in a mannitol-free PDA medium. In turn, seeds tested at the osmotic potential of -0.6 MPa performed better than the other treatments due to the shorter time they were kept under the testing conditions. In this case, seed imbibition occurred only partly, and the germination proceeded, producing results even better than those of the control treatment.

The high emergence rate observed in the non-inoculated seeds by the 21st DAS was already expected because they, unlike the other treatments, were not impaired by the fungus. On the other hand, a rather expressive reduction was verified in seedling emergence at 1.2 MPa, probably due to the longer exposition to the pathogen (144 h). This fact contributed to intensifying the infective process of the seeds, a conclusion supported by the sanitization testing (Figure 2).

Barrocas et al. (2014) proved that, by adding mannitol at 0,8 MPa in a PDA medium, the number of cotton seedlings tended to drop as the exposure time to that substrate prolonged. Meneses et al. (2011) used the same technique to

evaluate cotton seed germination under water stress, but they employed different concentrations of another osmotic agent, PEG 6000. They realized that seed viability and seedling vigor got compromised up from 0.4 MPa.

However, it is worth noticing that the seeds inoculated under no water restriction (control treatment) and at the osmotic potentials of 0.6 MPa, 0.8 MPa and 1.0 MPa also exhibited high infection indices, as evidenced by the percentage of abnormal seedlings attained in the germination test (Figure 1). By contrasting the results of emergence and germination, it became clear that the higher number of abnormal seedlings was scored by the latter test, probably because of the different conditions provided during the procedures – in this case, the germination settings were more favorable to the fungus growth. In similar studies, Machado et al. (2004) and Araújo et al. (2006) also detected a significant reduction in cotton seedling emergence as the seeds remained longer in contact with the ramulose agent, in substrates with water restriction.

Conclusions

Employing mannitol as an osmosis-controlling agent at 1.0 MPa and 1.2 MPa does not significantly compromise *A. radicina* growth. Such conditions promote the highest infection rates and, consequently, the reduction of normal seedling emergence and the substantial increase in the number of abnormal ones. Remarkably, the osmotic potential of

1.2 MPa produces the lowest emergence rates after 21 days of sowing. Therefore, the use of mannitol in a PDA medium at 1.2 MPa is an efficient method for infecting carrot seeds with the fungus *A. radicina*, for either epidemiological purposes or seed health management.

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