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Resistance to Fusarium Wilt in watermelon accessions inoculated by chlamydospores

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ABSTRACT

The present study aimed to evaluate different inoculation methods of *Fusarium oxysporum* f. sp. *niveum* in watermelon and the reaction of accessions from this crop. Firstly, seven inoculation methods using conidia were tested on the susceptible cultivar Sugar Baby, including the standard dipping method. The methods initially tested were not efficient; therefore, we tested a new methodology using chlamydospores which are fungus survival structures. After the production of chlamydospores in vermiculite enriched with liquid culture media potato and sucrose (PS), the new method was tested on an experiment comparing inoculation by conidia and chlamydospores. For the conidia method, the plants were inoculated after their final leaf formed. For chlamydospores, inoculations were done in plants or sowed seeds. The use of chlamydospores in sowed seeds was effective for the inoculation of *Fusarium oxysporum* f. sp. *niveum* in watermelon and showed the highest severity scores in relation to the others methods. The inoculation method using chlamydospores also obtained the shortest means of root and shoot length in the cultivars Charleston Gray and Sugar Baby. Thus, using this methodology, 25 accessions from the watermelon germplasm were evaluated 21 days after inoculation with a grading scale. Eight accessions were classified as resistant, corresponding to 32% of accessions evaluated. Eight other accessions received the highest score of severity, proving the efficiency of the methology to evaluate the reaction to the disease.

1. Introduction

Watermelon cultivation is an activity practiced in different countries worldwide and it is a main vegetable grown in Brazil (FAO, 2015). This vegetable crop has relevant economic importance. Due to its short cycle, watermelon has attracted producers looking for quick financial returns. However, the main challenge in fruit cultivation is the occurrence of several diseases throughout the crop cycle (Romay et al., 2014).

Among the diseases that occur in watermelon is Fusarium Wilt, which is caused by the fungus *Fusarium oxysporum* f. sp. *niveum*. The pathogen produces two types of conidia, the macro and microconidia, and in the absence of a host, it can survive saprophytically for up to six years. The pathogen's survival is even more elongated and can be extended for more than 10 years when the production of survival structures, such as chlamydospores, occurs (Zhang et al., 2015). This is a common disease in several countries including the United States and China and it causes significant damage to watermelon production (Lü et al., 2011). The management of Fusarium Wilt has been a challenge to watermelon producers because it is caused by a soil pathogen. Because of this, producers have opted for planting in areas without disease occurrence, which is a less sustainable practice. The most common control method is chemical control, but there is a shortage of recommended products. Another commonly adopted measure is crop rotation, however, it becomes inapplicable when contamination occurs in the field due to the easy dissemination and survival characteristics of the pathogen. This makes genetic control, with the use of resistant cultivars, the most viable and efficient alternative for the management of Fusarium Wilt (Everts and Himmelstein, 2015).

In Brazil, the occurrence of Fusarium Wilt has been observed in fields of watermelon (Silva et al., 2016). However, there have been only a few studies in Brazil to evaluate the Fusarium Wilt resistance in cucurbits. The fungus inoculation is difficult and this is one of the reasons it is studied so rarely. All the methods used in several studies have used

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conidia, fungal reproduction structures, for inoculation.

The most used inoculation method on different *F. oxysporum* formae speciales has been dipping or the tray-dip method (Latin and Snell, 1986), which consists of the immersion of the roots in a suspension of conidia. Azevedo et al. (2015), using the dipping method of inoculation of *F. oxysporum* f. sp. *phaseoli* in bean genotypes, obtained variability for the reaction of the genotypes to disease. In addition, other studies prove that the dipping method has efficiency, such as on lettuce (Cabral et al., 2014) and tomatoes (Barboza et al., 2013).

An alternative to the standard method is inoculation using chlamydospores. The occurrence of disease in watermelon when planting in areas with disease registration could be related to the existence of these structures in the soil (Silva et al., 2016). According to Sanogo and Zhang (2016), the chlamydospores would be the primary inoculum under these conditions. However, there are no reports of the use of chlamydospores for evaluation of resistance in watermelon. Thus, the aim of this study was to evaluate different inoculation methods of *F. oxysporum* f. sp. *niveum* on watermelon using conidia and chlamydospores to identify the most efficient method to evaluate the reaction of Fusarium Wilt on watermelon accessions.

2. Materials and methods

2.1. Inoculation methodologies of F. oxysporum f. sp. niveum

The experiments were conducted in three steps in the laboratories and the greenhouse at the Federal University of the São Francisco Valley in Petrolina, Pernambuco, Brazil.

The seeds of the commercial cultivar Sugar Baby, which classified as susceptible for all races of *F. oxysporum* f. sp *niveum* (Zhou et al., 2010), were used for inoculation. The seeds were sowed on trays containing substrate for vegetables (compound of pine bark, peat, and expanded vermiculite enriched with macro and micronutrients). The trays containing seedlings were kept under a 50% shaded screen and irrigated daily until inoculation, which occurred 15 days after sowing.

The inoculations were carried out using a monosporic culture from one isolate of *F. oxysporum* f. sp. *niveum* (FON 10). The isolate was incubated in potato agar dextrose (PDA) and maintained in BOD at 25 °C for 10 days. After the growth, the conidial suspension was prepared with the addition of 10 mL of distilled water onto the plate and streaking using a Drigalski spatula. The conidial concentration in the inoculum suspension was adjusted using hemocytometer of Neubauer camera type to 1×10^6 conidia ml^{-1}.

The plants were inoculated when they presented three definitive leaves using seven methods (Table 1) and transplanted into 200 mL plastic cups containing commercial coconut powder substrate.

The evaluation occurred 45 days after inoculation (DAI) using the grading scale for hypocotyl lesions according to Dias et al. (2002). At the end of the experiment, the following development variables were evaluated: biomass weight, fresh root mass, fresh shoot mass, root dry mass and dry shoot mass.

2.2. Inoculation using chlamydospores and spores by the modified immersion method

The inoculum was obtained from the isolate FON10. First, the fungus was plated in Petri dishes containing PDA media. The plates were maintained under incubation in BOD at 25 °C for seven days. The liquid media PS (potato and sucrose) was used to produce the conidia (Dhingra et al., 2006). The PS media was prepared in Erlenmeyer 250 mL autoclaves for 15 min. After cooling, three mycelial discs about three millimeters in diameter were added into the PS media. Posteriorly, the Erlenmeyer was maintained with continuous rotation at 130 rpm at 25 °C for four days for the growth and sporulation of the fungus.

For the method using the chlamydospores, survival structures were obtained from an adaptation of the method proposed by Dhingra et al. (2006). In the present study, the chlamydospores were used with the objective of plant inoculation. The volume used to enrich the substrate and the incubation and drying times proposed by the authors were modified as follows: The substrate used for infestation was vermiculite enriched with PS media with the addition of two mL PS media for each gram of dry vermiculite. In total, 32 Liters of vermiculite were prepared and separated into 16 plastic bags with two liters of vermiculite in each bag. The bags were closed, homogenized and autoclaved for one hour on the first day and 30 min on the second day. The bags were refrigerated until a substrate infestation occurred four days after autoclaving.

The substrate was infested with an addition of 10 mL of conidial suspension per bag of vermiculite in an aseptic environment. The bags were closed for incubation and kept on benches at room temperature for 21 days. They were homogenized for better chlamydospore production. After this period, the bags were kept closed at temperatures between 26 and 30 °C for 19 days with paper towels to dry.

The inoculation tests were performed when the first definitive leaf developed for the two inoculation methods, in addition to the inoculation that occurred with the chlamydospore infested substrate method. The experiment was conducted with a completely randomized design with five replicates for each treatment and three inoculation methods: seedling with modified dipping method (TE1); seedling on substrate infested with chlamydospores (TE2); sowed seed on substrate infested with chlamydospores (TE3), further treatments on seedling transplant without inoculation (TE4) and sowed seed on non-infested substrate (TE5). The commercial cultivars Sugar Baby and Charleston Gray, plus the accession BGH 398 previously classified as resistant, were used in the inoculations tests.

The inoculated plants using conidia (TE1) were made according to modifications to the method proposed by Meru and McGregor (2016). The first modification was on the period of agitation for fungus growth in liquid media which was modified to four days. The second modification was the pot used for transplanting; in this study, disposable plastic cups of 200 mL containing expanded vermiculite were used. Cups with holes in their bases were kept inside the plastic tray containing a suspension of conidia for 30 min for substrate infestation by the fungus. The chlamydospore seedlings were transplanted onto substrate infested with chlamydospores (TE2) or seeds were sowed directly

Table 1
Inoculation methods tested for *F. oxysporum* f. sp *niveum* in watermelon.

Inoculation Methods	Description of Methods
Dipping with cut roots in conidial suspension	Wash the root system in water and cut 1/3 of the length with a subsequent dip in conidial suspension for 30 min
Dipping non-cut roots in the conidial suspension	Wash the root system in water, dip in conidial suspension for 30 min
Drop deposition of conidial suspension	Drop deposition of conidial suspension on plant stem followed by perforation
Injection of 0.1 mL conidial suspension	Inject conidial suspension by syringe in the stem of the plant with a volume of 0.1 mL
Digging and deposition of 10 mL of conidial suspension	Dig the substrate in the region around the hypocotyl with a pocketknife and deposit 10 mL of conidial suspension
Digging and deposition of 30 mL of conidial suspension	Dig the substrate in the region around the hypocotyl with a pocketknife and deposit of 30 mL of conidial suspension
Spray conidial suspension	Spray the conidial suspension on leaves until there is runoff

onto the infested substrate. After sowing, the plants were kept in the shade for four days and then taken to a greenhouse with a 50% shaded screen and irrigated daily. The evaluation occurred 22 days after inoculation using the grading scale according to Dias et al. (2002).

2.3. Watermelon accession inoculation to evaluate Fusarium Wilt resistance

The accessions evaluated were obtained from the Active Bank Germplasm of Cucurbitaceae in the Brazilian Northeast from Embrapa. Twenty five accessions were inoculated. The commercial cultivar Sugar Baby was used as a control and was sowed on non-infested substrate enriched with PS media. Five replicates were used for each treatment.

After sowing, the plants were kept in a room with continuous illumination for seven days. After this period, the plants were taken to a greenhouse with a 50% shaded screen and irrigated daily. The evaluation was performed 22 days after inoculation using a grading scale according to Dias et al. (2002). Accessions with average grades less than or equal to two were classified as resistant and accessions higher than two were classified as susceptible (Nascimento et al., 1995).

2.4. Data analysis

The data about the reactions of the inoculations for the first experiment were transformed using $\sqrt{x + 0.5}$ as the equation and the *F* Test at a 5% probability. Descriptive statistics and Anova were performed for the data of the development variables.

For the Fusarium Wilt reaction, the data obtained were analyzed for residue normality and homogeneity of variances by the Shapiro-Wilk and Bartlett (1937) tests at 5% significance, respectively. Data that did not meet the Anova premises were analyzed by the Kruskal-Wallis nonparametric test at a 5% probability. Statistical analyzes were performed using the statistical programs SISVAR (Ferreira, 2011) and R Core Team (2016).

3. Results

3.1. Watermelon inoculation methods for F. oxysporum f. sp. niveum

The inoculated plants presented initial lesions caused by the fungus *F. oxysporum* f. sp. *niveum*. However, the seven methods tested did not differ statistically from the control at a level 5% with the *F*-test (Fig. 1).

After proving that the lesions were caused by the inoculated fungus, the damage was measured. The vegetative growth data collected included biomass, fresh shoot mass, aerial shoot mass, fresh root mass and dry root mass. They were analyzed and no significant effect was observed by the *F*-test at a 5% probability for all methods studied and none of the methods differed statistically from the control (Fig. 2).

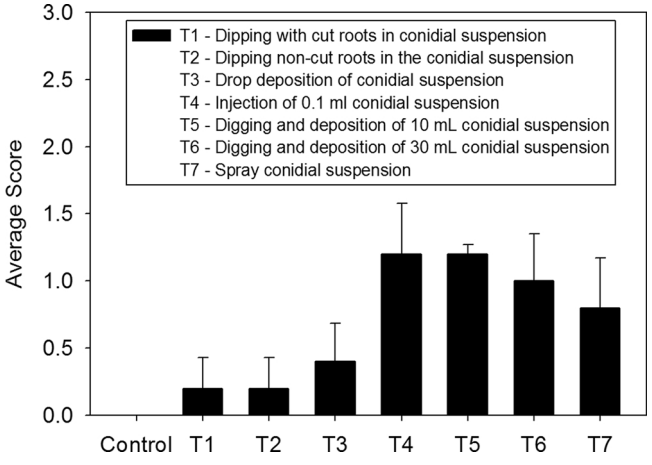

Fig. 1. Average grades for hypocotyl lesions after inoculation using conidia of *F. oxysporum* f. sp. *niveum* (FON10) and the seven inoculation methods on cv. Sugar Baby. Bars: standard deviation.

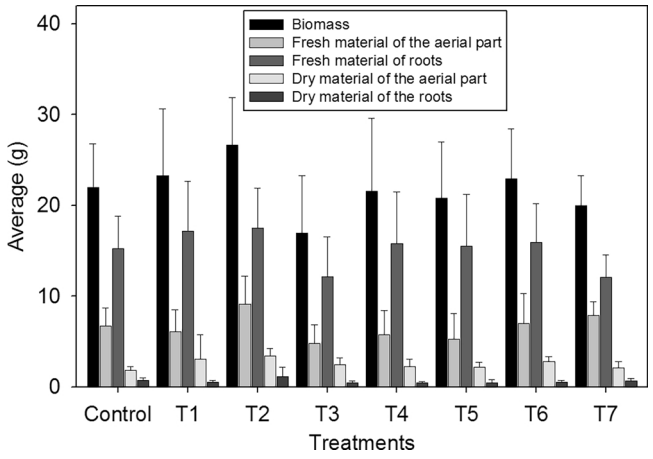

Fig. 2. Vegetative growth variables of watermelon plants inoculated with conidial suspension of *F. oxysporum* f. sp. *niveum*. The captions for this figure (T1–T7) are the same as Fig. 1. Bars: standard deviation.

3.2. Chlamydospore inoculation and the modified conidial dipping method in watermelon

Sowed seeds on substrate infested with chlamydospores of *F. oxysporum* f. sp. *niveum* showed the highest average scores for hypocotyl lesions observed on the three accessions of Sugar Baby, Charleston Gray and BGH 398 (Fig. 3). Both of the commercial cultivars Sugar Baby and Charleston Gray, when inoculated by this method, differed statistically from the others two inoculation methods by the Scott-Knott test at a 5% probability. There was no statistical difference in the average grades of reaction between the inoculation methods tested for the BGH 398 accession (Fig. 3). The dipping method with modifications obtained an intermediate grade between the three methods evaluated on cv. Sugar Baby.

There was significant interaction between accessions and inoculation methods for root length (Fig. 4). In the analysis of the accessions' impacts within each method, there was statistical difference only for the inoculation method of sowed seeds on infested substrate with chlamydospores. The cultivars Charleston Gray and Sugar Baby had the

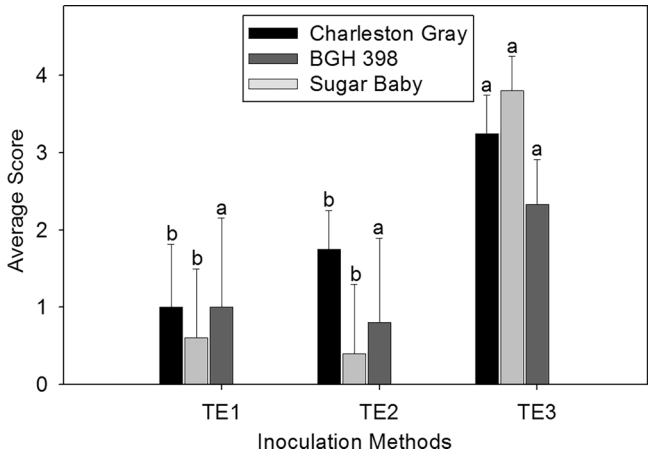

Fig. 3. Average grades of three inoculation methods evaluated with *F. oxysporum* f. sp. *niveum*: Seedling with modified dipping method (TE1), Seedling on infested substrate with chlamydospores (TE2) and sowed seed on substrate infested with chlamydospores in the commercial cultivars Sugar Baby and Charleston Gray and the accession BGH 398. Means followed by the same letter within genotype do not differ statistically by Scott-Knott at 5% probability.

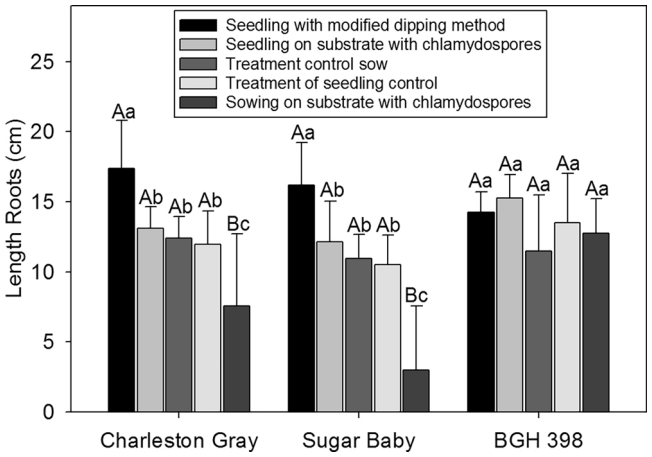

Fig. 4. Average root length of the commercial cultivars Charleston Gray and Sugar Baby and the accession BGH 398 inoculated with *F. oxysporum* f. sp. *niveum*. Means followed by a lowercase letter within the same genotype and uppercase letter between methods do not differ statistically by Scott-Knott at 5% probability. Bars: standard deviation.

shortest average length, differing statistically from BGH 398 by the Scott-Knott test at 5% probability. When analyzing method effects within accessions, the length did not differ statistically for the BGH 398 accession. For 'Sugar Baby' and 'Charleston Gray', the method of infested substrate with chlamydospores presented the shortest averages and the dipping method with modifications presented the longest averages of root length. In these two cultivars the means of the two methods were statically superior among methods tested at a level of 5% by the Scott-Knott test.

For the shoot length data, the interaction accessions and inoculation methods were not significant (Fig. 5). For the accessions, the 'Charleston Gray' cultivar had the highest mean and differed statistically from 'Sugar Baby' and BGH 398 (Fig. 5-A). The method of sowed seeds on substrate with chlamydospores provided the lowest shoot growth (Fig. 5-B), differing statistically from the others by the Scott-Knott test ($p < 0.05$).

3.3. Reaction of watermelon accessions inoculated with F. oxysporum f. sp. niveum chlamydospores on fusarium wilt resistance

Among the 25 accessions inoculated by the chlamydospores method, variability was observed in reactions by the accessions to Fusarium Wilt (Table 2). The reaction between accessions differed statistically by the Kruskal-Wallis test ($P < 0.05$). Of the 25 accessions inoculated, eight were classified as resistant to Fusarium Wilt (Table 2). Among the resistant accessions, most of them belong to the species *C. lanatus* var. citroides, such as the BGCIA 229 and BGCIA 962 which have an immune-like reaction. In the group of 17 classified as susceptible, eight accessions had scores of 4, the highest level of virulence.

Table 2
Mean scores, standard deviations (in parentheses) and reactions of watermelon accessions inoculated with *F. oxysporum* f. sp. *niveum* plus the commercial cultivar Sugar Baby used without inoculation of the fungus. The accessions were classified as resistant (R) and susceptible (S) to Fusarium Wilt. Accessions with average grades less than or equal to two were classified as resistant and accessions higher than two were classified as susceptible (Nascimento et al., 1995).

Accessions	Botanical Specie	Mean Score	Reaction
cv. Sugar Baby[a]	*Citrullus lanatus* var. *lanatus*	0.00 (+0.00)	*
BGCIA 229	***Citrullus lanatus* var. *citroides***	**0.00 (± 0.00)**	**R**
BGCIA 962	***C. lanatus* var. *citroides***	**0.00 (± 0.00)**	**R**
BGCIA 226	***C. lanatus* var. *citroides***	**0.20 (± 1.73)**	**R**
BGCIA 849	***C. lanatus* var. *lanatus***	**0.25 (± 0.00)**	**R**
BGCIA 227	***C. lanatus* var. *citroides***	**0.40 (± 1.34)**	**R**
BGCIA 812	***C. lanatus* var. *lanatus***	**1.00 (± 0.00)**	**R**
BGCIA 223	***C. lanatus* var. *citroides***	**1.20 (± 2.00)**	**R**
BGCIA 219	***C. lanatus* var. *citroides***	**1.67 (± 1.53)**	**R**
BGCIA 959	*C. lanatus* var. *lanatus*	2.20 (± 1.79)	S
BGCIA 225	*C. lanatus* var. *citroides*	2.40 (± 2.19)	S
BGCIA 821	*C. lanatus* var. *lanatus*	2.40 (± 0.45)	S
BGCIA 028	*C. lanatus* var. *lanatus*	3.00 (± 0.89)	S
BGCIA 115	*C. lanatus* var. *lanatus*	3.00 (± 0.00)	S
BGCIA 865	*C. lanatus* var. *lanatus*	3.20 (± 0.00)	S
BGCIA 843	*C. lanatus* var. *lanatus*	3.25 (± 0.00)	S
BGCIA 952	*C. lanatus* var. *lanatus*	3.33 (± 1.15)	S
BGCIA 036	*C. lanatus* var. *lanatus*	3.40 (± 2.19)	S
BGCIA 002	*C. lanatus* var. *lanatus*	4.00 (± 1.50)	S
BGCIA 012	*C. lanatus* var. *lanatus*	4.00 (± 0.50)	S
BGCIA 034	*C. lanatus* var. *lanatus*	4.00 (± 1.79)	S
BGCIA 040	*C. lanatus* var. *lanatus*	4.00 (± 0.00)	S
BGCIA 714	*C. lanatus* var. *lanatus*	4.00 (± 0.00)	S
BGCIA 811	*C. lanatus* var. *lanatus*	4.00 (± 1.15)	S
BGCIA 882	*C. lanatus* var. *lanatus*	4.00 (± 2.05)	S
BGCIA 947	*C. lanatus* var. *lanatus*	4.00 (± 0.00)	S

*Mean of the control treatment without pathogen inoculation.
[a] Susceptible cultivar Sugar Baby used as a control treatment without inoculation.

4. Discussion

4.1. Chlamydospore use on inoculation of F. oxysporum f. sp. niveum in watermelon

It was difficult to observe watermelon susceptibility to *F. oxysporum f. sp. niveum* in the initial tests of inoculation (Fig. 1). With the dipping method used in other studies, it was not possible to observe similar results (Cohen et al., 2014; Niu et al., 2016). The inefficiency of the method to produce symptoms of disease is a probable explanation for

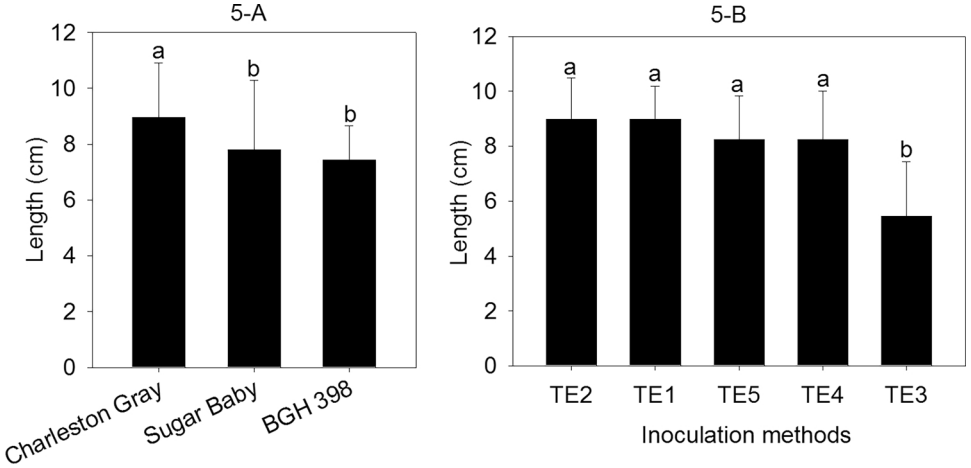

Fig. 5. Average shoot length of three genotypes (Fig. 5-A) inoculated by three inoculation methods plus the control treatments without inoculation (Fig. 5-B): seedling with modified dipping method (TE1); seedling on infested substrate with chlamydospores (TE2); sowed seed on substrate infested with chlamydospores (TE3), further treatments without inoculation with seedling transplant (TE4) and sowed seed on non-infested substrate (TE5). Means followed by the same letter do not differ statistically by Scott-Knott at 5% probability. Bars: standard deviation.

the scarcity of published works on this pathosystem in Brazil. It is worth mentioning that this experiment was performed twice. In the first experiment, no symptoms were observed (data not shown). The dipping method has been successfully used in other pathosystems in Brazil, such as in beans (Ribeiro and Hagedorn, 1979; Chiorato et al., 2015; Henrique et al., 2015), lettuce (Cabral et al., 2014), tomatoes (Barboza et al., 2013) and bananas (Amorim et al., 2009), in which virulence and aggressiveness of the pathogen were observed. Henrique et al. (2015) reported difficulties in obtaining a resistance reaction in bean genotypes due to pathogen aggressiveness using the dipping inoculation method. In the first experiment, with inoculation using conidia, even the cultivar Sugar Baby, which is susceptible to all races of *F. oxysporum*, showed no difference in the treatments compared to the control (non-inoculated) (Fig. 1). Thus, the methods tested were not efficient for the evaluation of *F. oxysporum* f. sp. *niveum* aggressiveness. When the other characteristics were evaluated, no interference of the methods in the development of the plants was observed during the evaluated period (Fig. 2).

Reports from producers and researchers working with watermelon breeding indicated the occurrence of this disease in planting areas. According to Silva et al. (2016), in a survey about the frequency of diseases in areas of watermelon production in the state of Pernambuco, *F. oxysporum* f. sp. *niveum* is one of the pathogens observed.

By comparing methods presented in this study and the dipping method with modifications, lower root development can be observed among the commercial cultivars in the plants inoculated with the sowed seeds on infested substrate method (Fig. 4). By using chlamydospores in the inoculation, the average length of the shoot was also statistically lower than the plants of the other methods (Fig. 5-B). The highest severity scores assigned correspond to the most aggressive methods in relation to the other methods. The inoculation method using chlamydospores also obtained the lowest means to root and shoot length in the cultivars Charleston Gray and Sugar Baby.

4.2. Reaction of watermelon accessions inoculated with F. oxysporum f. sp. niveum chlamydospores for Fusarium Wilt resistance

The inoculation method using chlamydospores was adequate for watermelon inoculation. Plants were observed in which pathogen aggressiveness was low, including accessions BGCIA 229 and BGCIA 962 (Table 2), until the occurrence of plant death which was observed in 48.8% of inoculated plants. From the eight accessions classified as resistant, two were *C. lanatus* var. *lanatus*, BGCIA 849 and BGCIA 812, and the other six were *C. lanatus* var. *citroides* which is the same variety of differentiating cultivar PI-296341-FR that was used in the races identification of *F. oxysporum* f. sp. *niveum* (Zhou et al., 2010). Santos et al. (2014), when studying rootstocks compatibility on watermelon, included the BGCIA 229 accessions as compatible among five *C. lanatus* var. citroides accessions with nematodes resistance. These authors also mention that BGCIA 229 would be an accession with potential Fusarium Wilt resistance which was confirmed in the present study. Another accession that has been used by researchers as rootstocks is BGCIA 962, which, according to Carvalho et al. (2013), is resistant to bacterial blight.

The existence of these Fusarium Wilt resistant accessions is an important tool for the development of commercial cultivars mainly resistant to *F. oxysporum* f. sp. *niveum* race 2, which are unavailable in the market (Cohen et al., 2014). In recent years, according to Ren et al. (2015), race 2 has been the most reported in studies aiming to find Fusarium Wilt resistance sources. According to these authors, Fusarium Wilt has been one of the main challenges to watermelon breeders. The cultivar Charleston Gray is classified as resistant to race 0 (Zhou et al., 2010). According to the results of the inoculation of this cultivar with chlamydospores in this study (Fig. 3), it can be observed that 'Charleston Gray' was susceptible to the FON-10 isolate. These results indicate that FON-10 is not race 0 of *F. oxysporum* f. sp. *niveum*. The present study developed efficient inoculation methods of *F. oxysporum* f. sp. *niveum* in watermelon. The use of chlamydospores in inoculation allows the identification of some sources of resistance to Fusarium Wilt and will contribute to advances in characterization studies of watermelon germplasm and identification of Fusarium Wilt resistance sources in Brazil that could be used by breeders and researchers.

5. Conclusions

The use of *F. oxysporum* f. sp. *niveum* chlamydospores for watermelon inoculation was effective. With the present method it was possible to identify different levels of Fusarium Wilt resistance on the inoculated accessions. Among the 25 accessions inoculated, eight were classified as Fusarium Wilt resistant to the FON-10 isolate.

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