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COMPARISON OF INOCULATION METHODS FOR SCREENING TOMATO, PEPPER, AND AUBERGINE PLANTS INOCULATED WITH *NEOSCYTALIDIUM NOVAEHOLLANDIAE*

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Abstract: *Neoscytalidium novaehollandiae* is a highly destructive fungus that causes serious yield losses in economically important crop plants. A reliable and rapid inoculation method for *Neoscytalidium novaehollandiae* was evaluated in eggplant, tomato and pepper seedlings under greenhouse conditions. Three different inoculation methods namely, cut-stem, spray, and injection were evaluated on tomato, pepper, and aubergine seedlings. The results showed that the cut-stem and injection methods were effective on tomato, pepper, and aubergine plants while the spray inoculation method was not found effective. Especially, the cut-stem inoculation method appears to be the most appropriate method for pathogenicity and screening of seedlings for *N. novaehollandiae* pathogenicity.

Keywords: *Neoscytalidium novaehollandiae*, Grafting techniques, Cut-stem, Spray, Injection

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1. Introduction

Tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annum* L.) and aubergine (*Solanum melongena* L.) are among the most commonly grown and consumed crops in Türkiye as well as in the world. The production and consumption of the fruits of these plants require high demand in the market. The amount of production is 13 million tons for tomato, 3.018.775 million tons for pepper and, 781.242 tons for aubergine (TÜİK, 2024). Many diseases and pests are encountered in the production process of these vegetables during cultivation, harvesting, and storage. In recent years, global warming has played an important role and made a significant impact on plant pathogens that has not been observed previously (Jamieson et al., 2012; Elad and Pertot, 2014). The impact of plant pathogens under environmental stress conditions increased radically and has caused more devastating effects on crop plants (Félix et al., 2016; Yan et al., 2018). For example, *Lasiodiplodia theobromae* is an opportunistic pathogen of woody plants. It produces cellulase, lignocellulase, 18 chitin-binding gene families, and heat shock proteins at high temperatures. It is evident that its virulence increases along with the increase in air temperature. The most important plant pathogens for commonly consumed vegetables are *Botrytis cinerea, Clavibacter michiganensis* subsp. *michiganensis, Pseudomonas corrugata, Erwinia carotovora* subsp. *carotovora, Phytophythora infestans, Alternaria solani*, and Tomato spotted wilt, tospovirus, *Sclerotinia sclerotiorum, Phytophthora capsici,* and Cucumber mosaic virus, etc. (Fernando et al., 2004; Agrios, 2005; Höfte, 2006; Yardımcı and Çulal Kılıç, 2009; Zitikaitė and Urbanavičienė, 2010; Zhao at al., 2013; Todorović et al., 2016; Babalola et al., 2017; Hua et al., 2018; Wolters et al., 2018). In recent years, *Neoscytalidium* spp. have caused significant crop losses in vegetables and trees (Türkölmez et al., 2019a; Türkölmez et al., 2019b; Dervis et al., 2020a; Dervis et al., 2020b; Güney et al., 2022a; Güney et al., 2022b). One species of *Neoscytalidium* is *Neoscytalidium novaehollandiae* (Pavlic et al., 2008). *Neoscytalidium novaehollandiae* (Pavlic) is a dematiaceous fungal species that causes diseases in a wide range of plant hosts. This species belongs to a group of slow-growing, asexually reproducing microfungi, commonly referred to as "dark molds" or "black fungi" (Crous et al., 2006). Their damages in terms of crop quality become more prevalent. The fungal agent belongs to the *Botrtosphaeriaceae* family and forms filamentous, coiled, and irregular hyphae. The fungus mycelia develops rapidly in culture, its color is light at first and turns into brown and black over a short period of time. It can form both arthroconidia and pycnids in the same culture (Crous et al., 2006; Pavlic et al., 2008). *Neoscytalidium* species are able to infect plants through

air, soil, seed, fruit, etc. (Türkölmez et al., 2019a). They could also infect human beings through sinuses, skin, nails, wounds, etc. (Crous et al., 2006; Machouart et al., 2013; Bakhshizadeh et al., 2014; Da Silva et al., 2016). The family *Botryosphaeriaceae* contains many important plant pathogens, most of which are named opportunistic pathogens (Chethana et al., 2016). The pathogens in this family have great defensive strategies. High genetic variability among the family species gives a broad selection range for the host preferences. Many species in the *Botryosphaeriaceae* family infect a wide variety of plant hosts (Jami et al., 2014; Garcia et al., 2021). It is still under evaluation that the range of hosts could increase due to the aggressive behavior of the pathogen. Enzymes and heat shock proteins are able to protect the fungus from being digested by host-triggered enzymes and metabolites (Paolinelli-Alfanso et al., 2016). The unique genomic structure enables the adaptation of the fungus to new ecological conditions. The genes protect the fungus by detoxifying phytotoxins during the infection stage (Chen et al., 2014; Yan et al., 2018). Thus, the fungus can easily spread around and live in high temperatures and other adverse conditions and create more pathogenicity. Quite a few reasons have been attributed to increased pathogenicity such as drought, high temperature and water stress as well as plant age, growth period and inoculation methods (Jordaan et al., 2019; Khamari et al., 2019; Chaturvedi, 2021). It is, therefore, clear that opportunistic pathogens have a high capacity to adapt to stressful conditions (Kılınc, 2021).

To evaluate the behavior of the pathogen in vitro and in vivo conditions, and the responses of plants, we need to find out a quick and reliable inoculation method that would reveal the characteristic of the pathogen in a very short time. In this study, three different inoculation methods such as spraying conidia through leaves, syringe inoculation through stem, and cut-stem inoculation were made on tomato, pepper and aubergine plants with *Neoscytalidium novaehollandiae* isolated from pistachio trees in Şanlıurfa province (Kılınc,2021).

2. Materials and Methods

2.1. Growth of Fungus

Tomato (*Solanum lycopersicum* L. cv. SC2121), pepper (*Capsicum annum* L. cv. Acıburun) and aubergine (*Solanum melongena* L. cv. Diyarbakır Karası) plants were inoculated with *Neoscytalidium novaehollandiae* isolate (NCBI registration number: OL455801) at the 4-6 true leaf stage. The isolate was kindly provided by the plant pathologist Şahinmerdan Türkölmez from GAP Research Institute in Şanlıurfa. PDA medium was used for culturing of *N. novaehollandiae* isolate. The medium was poured as 10 ml into sterile plastic petri plates (10 cm in diameter) and kept until it solidified under aseptic conditions. Mycelial discs (8 mm) were taken from the ends of the fully developed 5-8 day-old *N. novaehollandiae* culture and placed in the center of newly-prepared petri plates. Petri dishes were then incubated at 25 ±1°C for 6-8 days (Fig. 1). The fungal spore suspension was adjusted to 1×10^6 conidia ml^{-1} (Kee et al., 2017).

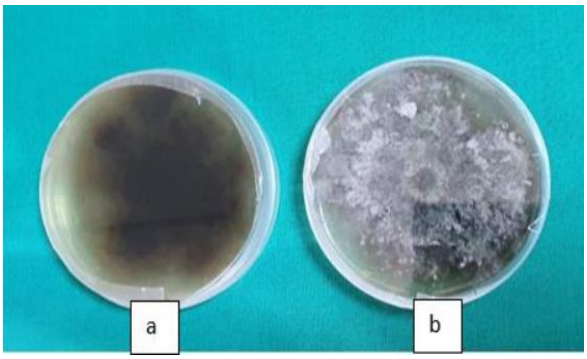

Figure 1. Appereance of mycelial growth of *N. novaehollandiae* a) back side; b) front side of the culture in PDA medium. The fungus culture gets darker during growth and development.

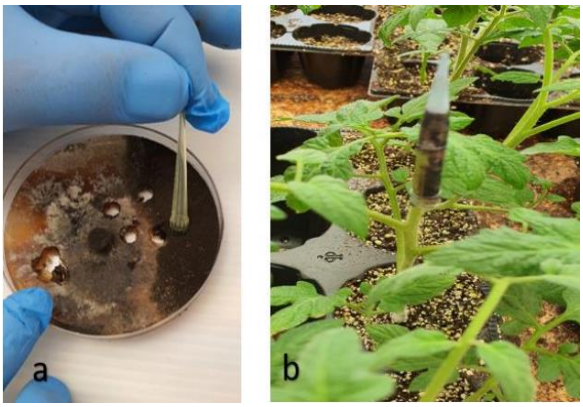

Figure 2. Cut-stem inoculation method using a 1 ml-pipette tip for the cut-surface area a) collection of mycelial discs via 1 ml-pipette tip b) pipette tip bearing fungal discs for the inoculation of cut-surface area.

2.2. Inoculation of Plants

A mixture of peat: perlite:sand (2:1:1) was prepared for the growth of tomato, pepper and aubergine plants in vials and the plants were maintained at 26 °C with 60% relative humidity at 14:10 h day: night photoperiod conditions in a greenhouse. Irrigation of the plants was made on demand.

2.2.1. Cut-stem inoculation method

The plants were cut with the help of a sterile scalpel, approximately 8 cm from the top, and the PDA discs bearing the fungus mycelia and the conidia were placed on the cut area. The cuts were protected by covering them with a 1 ml- pipette tip (Fig. 2). For the control group, sterile PDA discs were placed in these cuts and covered with a 1 ml pipette tip as shown below. A pipette tip is designed to keep the pathogen in infected tissues throughout the infection period and minimizes the pathogen escape and allows for a healthy comparison (Mengistu et al., 2007). The pipette tips were removed from the cut surface after 5 days and the progression of

the disease as brown discoloration alongside the longitudinal stem starting from the cut surface (downward) was measured for a period of 30 days at 5-day intervals. Using pipette tips for the cut-stem surface area of tomato, pepper, and aubergine plants is a new approach to increase the efficacy of mycelial or conidial inoculation. With this approach, a simulation for wounded tissues under humid conditions was created, which is one of the possible ways infection in nature (Twizeyimana et al., 2012).

2.2.2. Spray method

The fungal spore solution was prepared by pouring 10 ml sterile-distilled water onto the culture medium of *N. novaehollandiae* grown in PDA in petri plates. Then, the concentration of fungal conidia was arranged to 1×10^6 condia ml^{-1} under a microscope using a hemocytometer. The conidial suspension (5 ml) was then sprayed onto the leaves of tomato, pepper, and aubergine seedlings, Fig 3. Control plants were sprayed with sterile water only. Immediately after spray, each pot was covered fully with a transparent nylon bag and kept for 24 hours. At the end of the day, the bags were removed and the plants were evaluated symptomatologically for 30 days at 5-day intervals.

Figure 3. Spray inoculation of aubergine plants.

Figure 4. Injection of pepper plants.

Figure 5. Necrotic areas on leaflet margins.

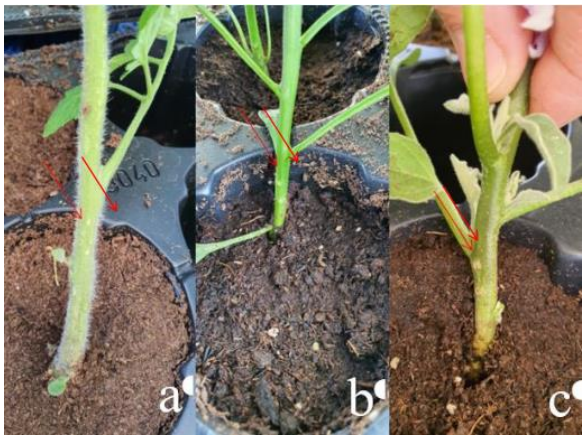

Figure 6. Necrotic spots appearing after N. novaehollandiae injecion on a) tomato b) pepper c) aubergine.

2.2.3. Injection method

The fungal spore solution (1×10^6 conidia ml^{-1}, 100 µl) was twice injected with a green-tipped syringe at 1 cm internals alongside the length of tomato, pepper, and aubergine seedlings approximately starting 3 cm above the soil surface (Fig. 4). For the control plants, 100 µl of sterile water was used (Kee et al., 2017). After the inoculations, the samples from the symptomatic plants were cut into small particles (2-5 mm) for a period of 30 days at 5-day intervals. The samples to be assessed were sterilized with 1% NaOCI solution for 45 seconds, and washed with sterile distilled water 3 times, then rinsed in 70% ethanol for 30 seconds. The samples were then dried on blotting papers. The measurements were made.

2.3. Disease Symptom Index

Plants were also scored for symptoms of a disease using the following scale (Dikilitas, 2003).

0-No symptoms

1-Trace of infection; yellowing visible symptoms at the cotyledon level,

2-Slight infection; chlorosis or yellowing patches affecting less than 50% of the leaves

3-Moderate infection; widespread symptoms such as chlorosis, wilting, necrosis, general decline in plants,

4-Severe infection; plant weak and stunted,

5-Extremely severe infection; only some green parts left in plants

6-Plants are completely dead

From these symptoms, a disease index was created and expressed with the folowing formula (i)

The number of plants showing a particular value (from 0 to 6) were multiplied by that value and the figure obtained for all plants summed and the total multipled by 100 to get % value. This value was divided by a maximum value of symtpms to get the ratio.

SI= Symptom index value
N= number of plants

$$\% SI = \frac{\sum SI}{6 \times \sum n} x100 \quad \text{(i)}$$

Table 1. The length of vascular discoloration and symptom index values of tomato plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm)/SI (%)	Spray method Vascular discoloration (mm)/SI (%)	Injection method Vascular discoloration (mm)/SI (%)
10	4 /10	0.1 /5	1 / 10
15	4/ 20	0.1 / 7	1 / 15
20	6 / 40	0.1 / 10	1 /25
25	8 / 60	0.1 / 10	1 / 30
30	12 / 65	0.1 / 10	1 / 35

SI= Symptom index value.

Table 2. The length of vascular discoloration and symptom index values of pepper plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm) / SI (%)	Spray method Vascular discoloration (mm) / SI (%)	Injection method Vascular discoloration (mm) / SI (%)
10	2 / 5	0.1 / 2	0.1 / 2
15	4 / 13	0.1 / 2	0.1 / 2
20	4 / 15	0.1 / 3	0.1 / 4
25	5 / 20	0.1 / 3	0.1 / 5
30	5 / 25	0.1 / 3	0.1 / 8

SI= Symptom index value.

Table 3. The length of vascular discoloration and symptom index values of aubergine plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm) / SI (%)	Spray method Vascular discoloration (mm) / SI (%)	Injection method Vascular discoloration (mm) / SI (%)
10	2 / 5	0.1 / 2	0.1 / 2
15	2 / 5	0.1 / 2	0.1 / 2
20	2 / 5	0.1 / 3	0.1 / 4
25	2 / 8	0.1 / 3	0.1 / 5
30	2 / 12	0.1 / 3	0.1 / 8

SI= Symptom index value.

3. Results and Discussion

Infection progress on tomato, pepper and aubergine plants was determined in terms of length (mm) in the vascular discolouration. Tomato, pepper and aubergine plants following inoculations with different methods showed that the symptoms of the disease progressed further with the increase of incubation time. However, the inoculation methods played significant roles about the progress of the disease. There were also differences in terms of disease responses among the plant species (Tables 1, 2, and 3). Infection speed in internal tissues in terms of vascular discoloration length showed that tomato plants were the most susceptible to *N. novaehollandiae* when compared to those of pepper and aubergine plants (Table 1). Inoculation after 20 days was found to be critical in plants with regard to disease progression. Infection progress in pepper and aubergine plants was prevented with the formation of callose tissues around the inoculation points and the infection area did not move on further when compared to the previous days. Aubergine plants were the most resistant plant that the infection rate remained still throughout the course of the experiment. The vascular discoloration length in thespray inoculation method remained constant at 0.1 mm for 30 days in all plants (Table 2). In some leaflets, necrotic areas and yellow patches were evident. However, this inoculation method was not found effective. Symptom index values even did not increase remarkably. When reisolation was made from those of the infected plants, the pathogenic agent was only recovered from the leaves of tomato plants exhibiting symptoms of the disease. In the injection method, the stems of the plants were more infected with 100 ml of the spore solution 1×10^6 spor (ml) through the stems. As a result, 1 mm of necrosis occurred around the inoculation site in tomato plants. However, aubergine and pepper plants formed new callus tissues around the infection sites and confound the pathogen throughout the experimental period. Reisolation was made from tomato plants while other plants gave negative results. Symptom index values significantly increased only in tomato plants. Cut-stem method was found to be the most effective method compared to those of other methods employed in this study for the determination of pathogenicity of *N. novaehollandiae* on tomato, pepper, and aubergine. This method could be suggested as a method to test the resistance level of vegetables. Although the efficient (cut-stem) method tested here was evaluated previously by other researchers on different fungi, to our knowledge no such comparison was made

on tomato, pepper, and aubergine with *N. novaehollandiae* in greenhouse conditions. Although the spray and the injection methods have been widely used in quite a few studies in different plant types in greenhouse conditions, however, callose accumulation around the infection sites following injection may retard the disease development if the germplasms are relatively/moderately tolerant or resistant to diseases. In the spray inoculation method, although a plastic bag was used to cover up the plants following inoculations, however, the low hummidity in greenhouse conditions might have prevented the disease development internally. The progress of disease symptoms in terms of internal browning has also been reflected in symptom index values taken at 5 days internally until the end of the experimental period. One of the main advantages of this technique compared to other inoculations methods used here is that a uniform amount of inoculum is placed directly onto the cut-stem paranchymatic tissues, which minimizes potential infection escapes (Ćuk et al., 2022). They stated that the method significantly revealed the resistance levels of sunflower plants to *Macrophomina phaseolina* (Ćuk et al., 2022). This method could be further evaluated with different fungi having different spore suspensions to enable precise quantitative inoculation which would help reduce experimental error. Since continuous inoculation is enabled with the cut-stem inoculation method, the resistance levels of inoculated plants could easily be determined without letting no interaction between the environmental stress agents and the pathogens. The whole experimental process could be completed over a month, in fact, first 2 weeks would indicate the resistance levels of inoculated plants. Since pathogens have no place to escape, it directly meets the paranchymatic tissues after inoculation even if variations existed in virulence levels. We could directly assess the resistance of plants as well as the virulence or pathogenicity of the fungal agent. Other environmental factors are significantly minimized. The cut-stem technique could serve as the most appropriate technique especially for new pathogens or new germplasms. Screening is going to be easier due to its ease of application. For example, Talapov (Talapov, 2020) stated that cut-stem inoculation technique was proven to be responsible on soybean plants inoculated with *Macrophomina phaseolina*. Due to easy applications of the inoculations method. Ćuk et al. (2022) suggested that the cut-stem method in sunflower plants could potentially be used in field trials and it could be a valuable tool in sunflower breeding for resistance to *M. phaseolina*. Twizeyimana et al. (2012) stated that there were several advantages of using cut-stem inoculation technique over to other inoculation methods. They reported that a uniform amount of inoculum placed onto an identical infection court via this technique minimized potential inoculum escapes. They also reported that the progress of the disease could be measured uniformly by measuring the extent of necrosis. Evaluation of this method could be completed within two weeks.

4. Conclusion

We propose that the cut-stem inoculation method applied to tomato, pepper, and aubergine is easy to apply and marks well and differentiates plant responses efficiently. However, one should notice that the rapid disease development that might result in plant death after 10-15 days of inoculation in vegetables can lead to misinterpration in germplasm studies. In this study, a preliminary investigation was made how the fungal agent *N. novaehollandiae* infected the host tissues such as tomato, pepper and aubergine plants via different routes of inoculation methods. Determination of the effectiveness of these methods could facilitate the selection of an appropriate inoculation method and screening the plant species in a very short time. For the selection of inoculation methods, we could prefer the cut-stem method over to spraying and injection methods. The cut-stem method is found the most efficient method on vegetable plants. Similar findings were also made by Talapov et al. (2021) who stated that the cut-stem method in sunflowers appeared to be the most appropriate for the pathogenicity of *Marcrophomina phaseolina* under controlled conditions. Since the fastest disease development on sunflowers was obtained with the cut-stem inoculation method. They stated that the cut-stem inoculation method yielded consistent results in pathogenicity and a variety of reaction studies. The most sensitive plant against *N. novaehollandiae* was found to be tomato and the most resistant plant was found to be aubergine. Data from this study will constitute important data for future studies, as no previous study for this pathogen has compared different inoculation methods.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	H.A.	F.D.	M.E.G.	M.D.
C	30	20	20	30
D	50			50
S	50			50
DCP	50	50		
DAI	40			60
L	60	40		
W	80			20
CR	30		20	50
SR	50		20	30
PM	20		40	40
FA		30	70	

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management. FA= funding acquisition

Conflict of Interest

The authors declare that they have no financial interests or relationships that could potentially lead to a conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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