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Induction of coffee wilt disease infection using different types of contaminant in field conditions in Democratic Republic of Congo

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A study was conducted on the induction of coffee wilt disease (CWD) infection with different types of contaminants using a randomized complete block design with four replications. Coffee seedlings were inoculated with infected bark, sub-bark tissues, wood necrotic and an artificial inoculum. Statistical analysis did not show significant differences (P>0.05) between treatments. Results obtained show that all contaminants used induced main symptoms of CWD. Chronologically, wilting appeared an average 55 DAI, followed by leaf browning (60 DAI), defoliation (69 DAI), leaf drying (82 DAI) and seedlings mortality (86 DAI). The lowest rate of wilting and leaf browning (11.1%) was recorded on seedlings inoculated with infected bark, and the highest rate (33.3%) was observed on seedlings inoculated with wood necrotic. Seedlings inoculated with sub-bark tissue expressed 11.1% of leaf drying, and those inoculated with an artificial inoculum presented 22.2% of mortality. Seedlings inoculated with sub-bark tissue expressed 22.1% of defoliation, while those inoculated with artificial inoculum expressed 39.4% of defoliation. The presence of *Fusarium xylarioides* was confirmed in dead woods of seedlings inoculated with sub-bark tissue, and those inoculated with artificial inoculum. Results obtained confirm the potential danger of wood debris from infected coffee trees, which can act as a source of infection and promote the spread of CWD when dragged through plantations.

Key words: *Coffea canephora*, coffee wilt disease, *Fusarium xylarioides*, field infection, types of contaminants, Democratic Republic of Congo.

INTRODUCTION

Coffee represents one of the most important agricultural commodities, ranking second in international trade after crude oil (Mishra and Slater, 2012). In Democratic Republic of Congo (DRC), coffee represents both an industrial and export product, which promotes the jobs creation and allows for currency inflows to the treasury

(Anonymous, 1998). However, its production is experiencing a remarkable decline due to aging plantations, the degeneration of planting materials, use of uncertified plant materials and attacks of pests and diseases (Tshilenge-Djim et al., 2004). Among these, coffee wilt disease (CWD) is one of the most important diseases dramatically limiting coffee production (Coste, 1989; Tshilenge-Djim et al., 2004; Girma et al., 2005; Sihen et al., 2012, 2013). This fungal disease caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim & Saccas) had devastated plantations in West Africa and also in DRC (Tshilenge-Djim et al., 2004).

In general, coffee plant infected by CWD shows no sign outside to notice the presence of the pathogen. This disease is manifested by a sudden stop of vegetation, the terminal buds of young shoots turn black; young leaves present along the main veins, chlorotic bands which reach quickly the entire limb. All leaves of terminal shoots turn yellow become flaccid, dangling towards the floor, turn brown, then darkening ensues with curling. The young shoots that carry them blacken also (Steyeart, 1948; Tshilenge-Djim et al., 2004). The disease spreads a few days after appearance of the first symptoms. In most cases, the first symptoms appear unilaterally on a single branch, the ends of the leaves wither, and soon after shoots, twigs and the entire plant, but the rest of the shrub seems intact. With older plants, this kind of phenomenon can last for several weeks, even several months (Saccas, 1951; Tshilenge-Djim, 2007).

In young plants, the appearance of external signs is occurs in a short time; this can be explained by the incubation period of the disease ranging from six to ten days as opposed to the older coffee trees. This means that this parameter is based on the volume of tissue of infected plants (Saccas, 1951; Tshilenge-Djim, 2007). Inside, the bark becomes dry and adheres to wood, deeper it turns brown then necrosis. Woody tissues appear gray in places and the central cylinder presented pink lines purplish or blackish blue mainly near the cambium. Drivers vessels are congested, many mycelial hyphae are colorless and tylose clogging. The vessel occlusion to the periphery and at the level of cortical crevices becomes complete (Saccas, 1951; Tshilenge-Djim, 2007). Having disappeared after the strict implementation of control strategies, CWD resurgence has been reported in the DRC where it continues to cause damage in the coffee growing areas of the Eastern and North Kivu provinces (Katenga, 1987; Mfwidi-Nitu, 1994) and affects up to 90% of plantations (Flood, 1996). In agronomic practice, solving phytosanitary problems lies not only in-depth knowledge of the host plant, its environment and the conditions of its culture, but also and especially on the pathogens and conditions of pathogenesis (Semal, 1989).

The presence of a primary inoculum in a field constitutes one of the most important factors in the spread of an epidemic disease. In the case of CWD, Kalonji and Onyembe (1996, unpublished data) mentioned the possibility of infection and propagation by different woods of infected coffee tree. These authors showed that control methods are not strictly respected by farmers. The farmers usually leave tracts of sick coffee on the ground or carry them through the plantations to their homes for domestic use: hedges of plots or firewood. It is in this context that the present study aimed to determine the role played by this parasitic wood in the infection of CWD. This study was initiated through different types of inoculum resulting from the fragments of the wood harvested on *Coffea canephora* tree infected with CWD.

MATERIALS AND METHODS

Description of experimental site

The study was conducted in the Experimental Garden of the Department of Biology, Faculty of Sciences, University of Kinshasa, DRC. The geographic coordinates recorded with the GPS (extrex Summit Garmin) indicated 4°19'S latitude, 15°8'E longitude, and 330 m of altitude. The experimental site falls within the Aw4 climate type according to Köppen classification characterization with 4 months of dry season (from second mid-may to first mid-September) coupled with 8 months of rainy season (from second mid-September to first mid-may) sometimes interrupted by a short dry season in January/February. Daily temperature averages 24.5°C and accuses small variations, and the annual rainfall is close to 1500 mm. The July and February are respectively the coldest and warmest month. The relative humidity is highest in April and May, and is minimum in September and October (Makoko and Mananga, 1986; Makoko et al., 1992). Data related to climatic conditions prevailing during the field trial are reported in Table 1.

The rainy season (October – November) corresponding to the period of intense flow of raw sap was favorable to the growth of the fungus, and promotes the transport of the fungus in vessels of plant.

Coffee seedlings used in the study

The plant materials used in the present study were 6 months old, obtained from seeds of *C. canephora* var *robusta* harvested in Research Station of Kiyaka (Institut National pour l'Etude et la Recherche Agronomiques/INERA) in DRC. The coffee genotype used in this study was identified as I1010203/OG. After germination, coffee seedlings were transplanted into polyethylene bags of 15 x 35 x 0.05 cm filled with forest soil from the valley of the Monastery Prieuré Notre Dame de l'Assomption. The soil used was characterized by dark brown coloration according to Munsell scale

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Table 1. Temperature, relative humidity and rainfall conditions prevailing during the experimental period.

Month	Temperature (°C)	Relative humidity (%)	Rainfall (mm)
August (2004)	27.8	76.0	17.6
September (2004)	28.2	76.0	20.1
October (2004)	28.7	80.6	183.4
November (2004)	28.6	81.3	248.7
December (2004)	27.7	89.6	266.6
January (2005)	30.5	98.9	24.0
February (2005)	26.7	81.4	27.5

Source: Department of Physics and Soil Hydrology (Regional Center of Nuclear Studies of Kinshasa: CREN-K).

Table 2. Interval of time (days) between coffee seedlings inoculation and the expression of CWD symptoms.

Contaminant	Mean time (day) of CWD symptoms expression (Mean±SD)				
	Wilt	Browning of leaves	Drying of leaves	Defoliation	Mortality of seedlings
Bark	17±2.8	22±1.2	N.O	50±2.4	N.O
Sub-bark tissue	51±1.7	56±3.4	61±2.5	58±2.3	66±2.4
Wood necrotic	60±2.4	65±3.5	N.O	66±2.5	N.O
Artificial inoculum	93±3.5	98±3.7	103±2.5	100±2.5	105±2.7
Control	N.O	N.O	N.O	45±1.2	N.O
LSD (0.05)	N.S	N.S	N.S	N.S	N.S

N.O: Not observed; N.S: not significant; SD: standard deviation.

(Anonymous, 2000), high porosity and a pH of 5.1. The polyethylene bags containing coffee seedlings were placed under a natural shade of *Acacia* spp. and *Eucalyptus* sp. Seedlings were watered every two days during dry periods.

Types of inoculum used

In the present study, natural and artificial inoculum were used. The natural inoculum was obtained from the infected coffee tree harvested in Yeboka region (province of Equateur, DRC), and consisted to wood shards of 4 x 3 x 0.5 mm taken in bark (colonized by perithecia), sub-bark tissue and wood necrotic. An artificial inoculum was isolated from a sample of the same infected material and purified in the Unit of Phytopathology laboratory (Faculty of Agronomy, University of Kinshasa, DRC), where it is stored as parent-strain in test tubes containing the synthetic nutrient agar (SNA) medium under paraffin. The SNA medium used constituted of: KH_2P0_4: 1 g; KNO_3: 1 g; $MgSO_4.7H_2O$: 0.5 g; KCl: 0.5 g; glucose: 0.2 g; sucrose: 0.2 g; agar Merck®: 20 g; and distilled H_2O: 1000 ml) (Tshilenge-Djim et al., 2004, 2011). Artificial inoculum used consisted of a pellet of 5 mm in diameter cut with a sterile scalpel blade outskirts of the mycelium of strain-girl previously obtained on SNA. This inoculum was taken where high concentration of conidia was previously observed upside of the Petri dishes under microscope (Olympus BX 40).

Technique of inoculation

The inoculation was done by technique of incision as described by Tshilenge-Djim et al. (2011), and consisted of inserting the inoculum into a notch made at the base of the stem of plant. The shards of infected tree representing different types of natural inoculum, and the stem of seedlings were first superficially disinfected with 70% ethanol which was allowed to evaporate for 10 min. The incision was made using a sterile scalpel blade at 1 cm below the insertion point of cotyledonary leaves in the plane of the first pair of true leaves; then, inoculum was inserted into incision and maintained in place by a ligature made with parafilm.

Experimental design, data recorded and statistical analysis

The study was performed using a randomized complete block design (RCBD) replicated four times using five treatments and nine seedlings per plot. Each treatment represented a type of contaminant, and non-inoculated seedlings were used as control. Seedlings inoculated were observed every 7 days during 4 months. Data collected were focused on the following variables: the time (days) between inoculation and the appearance of symptoms of CWD. The rate of each symptom was also recorded. The height and collar diameter of seedlings were measured, respectively with a ruban meter and a caliper. At the end of the trial, the re-isolation of the pathogen was made to check Koch's postulate. Data collected were submitted to analysis of variance (ANOVA) using R (R-2.12.0) software. Means comparison was performed by LSD test at 5% of probability level.

RESULTS

Data related to the interval of time (days) between inoculation and expression of symptoms of CWD are reported in Table 2. Results related to the rate of

Table 3. Rate (%) of different symptoms of CWD after inoculation with different contaminants.

Contaminant	Rate (%) of symptoms of CWD (Mean±SD)				
	Wilt	Browning of leaves	Drying of leaves	Defoliation	Mortality of seedlings
Bark	11.1±0.5	11.1±1.2	0	24.0±2.1	0
Sub-bark tissue	11.1±0.6	11.1±0.5	11.1±1.5	22.1±1.5	11.1±2.5
Wood necrotic	33.3±1.4	33.3±2.5	0	31.7±2.3	0
Pellet of SNA	22.2±1.2	22.2±2.4	22.2±2.0	39.4±2.1	22.2±1.6
Control	0	0	0	38.8±1.5	0
LSD (0.05)	N.S	N.S	N.S	N.S	N.S

N.S: Not significant; SD: standard deviation.

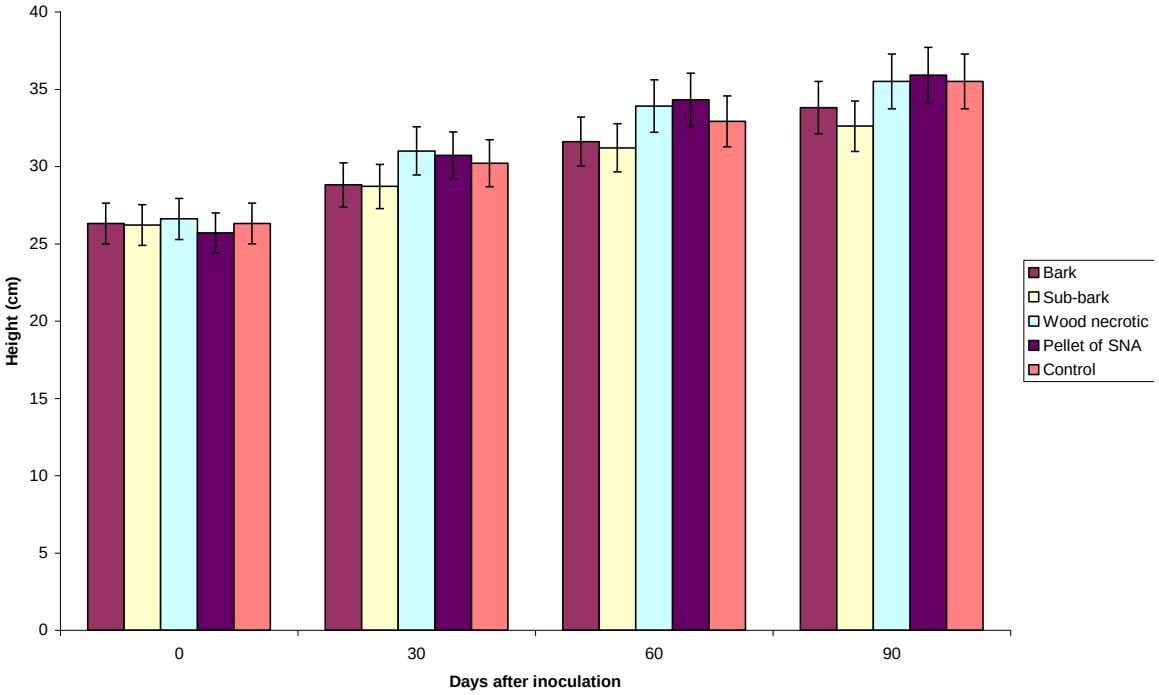

Figure 1. Height of coffee seedlings inoculated with different contaminants of *F. xylarioides*

Table 4. Presence/absence of *F. xylarioides* in dead woods.

Contaminant	Presence (+) or absence (-) of *Fusarium xylarioides* in dead woods
Bark	-
Sub-bark tissue	+
Wood necrotic	-
Pellet of SNA	+
Control	-

symptoms of CWD are presented in Table 3. Figures 1 and 2 illustrate the vegetative development of coffee seedlings. Results of the re-isolation of *F. xylarioides* are reported in Table 4.

Assessment of the pathogenicity of different contaminants used

Data reported in Table 2 show that wilting, leaf browning

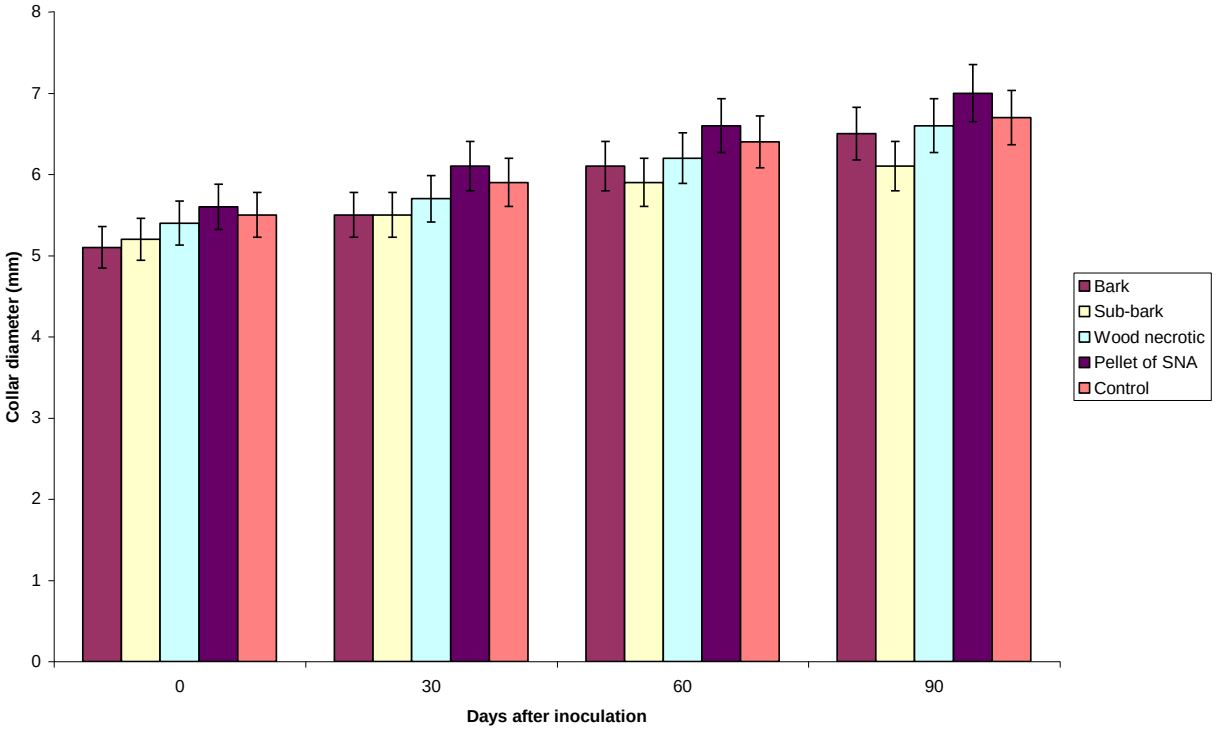

Figure 2. Collar diameter of coffee seedlings inoculated with different contaminants of *F. xylarioides.*

and drying, defoliation and seedlings mortality were main symptoms of CWD observed in inoculated seedlings. Seedlings of the control plot expressed only symptom of the defoliation. Statistical analysis did not show significant differences (P>0.05) between treatments. The wilting was recorded at an average 17 days after inoculation (DAI) on seedlings inoculated with infected bark, followed by leaf browning and defoliation at 22 and 50 DAI, respectively. The same symptoms were recorded an average at 93, 98 and 100 DAI, respectively, on seedlings inoculated with artificial inoculum. The leaf drying and seedlings mortality were recorded at an average of 61 and 66 DAI, respectively, on seedlings inoculated with sub-bark tissue, and an average of 103 and 105 DAI on seedlings inoculated with artificial inoculum.

Rate (%) of different symptoms of CWD

Results in Table 3 show that the percentage of inoculated seedlings expressing CWD symptoms did not vary significantly (*P>0.05*) between treatments. The lowest rate of wilting and leaf browning (11.1%) was recorded on seedlings inoculated with infected bark, and the highest rate (33.3%) was observed in seedlings inoculated with wood necrotic. Seedlings inoculated with sub-bark tissue expressed 11.1% of leaf drying, and those inoculated with an artificial inoculum presented 22.2% of mortality. The lowest rate of defoliation (22.1%) was recorded in seedlings inoculated with sub-bark tissue, while the highest rate (39.4%) was noted on seedlings inoculated with artificial inoculum.

Assessment of vegetative development of inoculated seedlings

Analysis of variance related to data presented in Figures 1 and 2 did not show significant differences (P>0.05) between inoculated seedlings. In Figure 1, thirty DAI, the highest height (31 cm) was recorded on seedlings inoculated with wood necrotic, while the lowest height (28 cm) was recorded on seedlings inoculated with infected bark, and sub-bark tissue. Sixty and ninety DAI, seedlings inoculated with artificial inoculum expressed the highest height (34.3 and 35.9 cm, respectively), while seedlings inoculated with sub-bark tissue expressed the lowest height (31.2 and 32.6 cm, respectively), compared to other seedlings. According to Figure 2, thirty DAI, seedlings inoculated with artificial inoculum expressed the highest collar diameter (6.1 mm), while seedlings inoculated with infected bark, and sub-bark tissue expressed the lowest collar diameter (5.5 mm). Sixty and ninety DAI, the highest collar diameter (6.6 and 7.0 mm, respectively), was recorded on seedlings inoculated with artificial inoculum, while the lowest value (5.9 and 6.1 mm, respectively), was noted on seedlings inoculated

with sub-bark tissue.

Re-isolation of *F. xylarioides* on dead woods

According to results presented in Table 4, the presence of *F. xylarioides* was confirmed in dead woods of seedlings inoculated with sub-bark tissue, and those inoculated with artificial inoculum.

DISCUSSION

Coffee wilt disease (CWD) is one of the main factors constraining coffee production in DRC. This fungal disease attacks all commercial *Coffea* spp. at any growth stage (Sihen et al., 2012). Results obtained in the present study show that all contaminants used are capable of inducing the following symptoms: wilting, leaf browning and drying, defoliation and seedlings mortality. Those symptoms were earlier described by Coste (1989) and Tshilenge-Djim et al. (1998, 2004) such as main symptoms of CWD.

Chronologically, wilting appeared at an average of 55 DAI, followed by leaf browning (60 DAI), defoliation (69 DAI), leaf drying (82 DAI) and seedlings mortality (86 DAI) (Table 2). The same trend was earlier reported by Tshilenge et al. (2010) and Tshilenge-Djim et al. (2011) who mentioned that CWD symptoms varied in their nature and their chronological sequence from the time of their appearance. The time of onset of CWD symptoms varies from one contaminant to another. The initiation of disease begins with wilting that is early on seedlings inoculated with infected bark, while it is late on seedlings inoculated with pellet of SNA. On inoculated seedlings, leaves turned brown at an average of 5 days after wilting, and the defoliation appeared at an average of 1-2 days after leaf browning on seedlings inoculated with wood necrotic, sub-bark tissue and a pellet of SNA. In general, when different contaminants induced the same symptom, it appears early on seedlings inoculated with infected bark, followed by sub-bark tissue, wood necrotic and pellet of SNA (Table 2).

According to various authors such as Sihen et al. (2012) and Muengula-Manyi et al. (2016), coffee plants infected with CWD always end up dying. In the present study, the mortality of seedlings was observed at an average of 12-15 days after wilting. Results reported by Tshilenge-Djim et al. (2011) showed that the wilting appeared at 16-17 days after inoculation, and the seedlings mortality appeared at 4-6 days after wilting. This difference can be due to the age of seedlings and pathogenicity of *F. xylarioides* strains used in those different studies. It is clear that mortality early appears when seedlings are youngest or when pathogen strain is more aggressive. In addition, according to Saccas (1951) cited by Tshilenge-Djim (2007), in young plants, the appearance of external signs of CWD is observed in a short time as compared to older coffee trees, and can be explained by the incubation of the disease which ranges from six to ten days. This means that this parameter is based on the volume of tissue of infected plant. By analyzing results recorded on the rate of symptom onset, it is possible to understand that there is no relationship between the types of contaminant and the degree of development of different symptoms.

The leaf drying and mortality were observed on seedlings inoculated with sub-bark tissue, and with pellet of SNA. The defoliation observed on control seedlings can be due to the senescence of leaves or other physiological cause. The absence of mortality on seedlings inoculated with infected bark bearing perithecia and with wood necrotic would be due in part to the identity of these perithecia which may be other than of *G. xylarioides*, capable of causing common symptoms of CWD except mortality. Indeed, Tshilenge-Djim et al. (2004) indicated that *F. solani*, *F. stilboides* and *F. falciforme* species are able to induce main symptoms of CWD without causing death of inoculated plant. In Ethiopia where CWD occurs, Girma (2004) isolated six fungal species belonging to the genus *Fusarium* from infected coffee samples. In Kenya, Baker (1972) indicated that *F. solani* and *F. oxysporum* induced varying types of wilting on coffee. In view of results reported by these many authors, it is possible that bark and wood necrotic tissues used in the present study were colonized by *Fusarium* species than *F. xylarioides*, which would explain the absence of mortality on seedlings inoculated with these contaminants. Results illustrated by Figures 1 and 2 revealed that during the trial, different contaminants did not influence the vegetative development of inoculated seedlings as compared to the control. Our observation corroborates findings reported by Muengula-Manyi et al. (2016), which revealed that inoculation of *Gibberella xylarioides* did not significantly influence the vegetative development of coffee seedlings.

Conclusion

The present study demonstrated that the different contaminants used induced characteristic symptoms of CWD at varying moment and degrees. Results obtained confirm the potential danger of wood debris from infected coffee trees, which can act as a source of infection and promote the spread of CWD when dragged through plantations. The use of shards of infected tissue taken at depth may constitute an alternative to culture on agar medium for inoculation under conditions where microbiological manipulations are not possible. The role of *F. xylarioides* in the pathogenesis of CWD was confirmed in the case of inoculation with sub-bark tissue and with pellet of SNA, whereas in case of seedlings that did not die, the potential role of other species of *Fusarium*

was suggested. The inoculation has not influenced the vegetative development of inoculated seedlings.

Conflict of Interests

The authors declared that there is no conflict of interests.

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