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In vitro selection of tomato plants resistant to *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahliae*

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Abstract

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Water scarcity, low and high temperatures, pests, and diseases harm the quality and yield of tomato production worldwide. To meet the increasing demand, it is necessary to develop new high-yielding varieties that combine disease resistance and fruit quality, suitable for cultivation in diverse climatic conditions. The present study aimed to create resistance in tomato plants against soil-borne fungal pathogens, *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahliae,* through an *in vitro* cell line selection approach. Cotyledonary explants of two tomato variants (Ideal and L1620) were tested to obtain resistant shoots to pathogens *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahliae*. The cotyledon explants were cultured on Murashige and Skoog basal medium supplemented with 2,2 mg/L kinetin and 1.6 mg/L IAA. To the medium were added three concentrations – 30% v/v, 40% v/v, and 50% v/v of the culture filtrates from two strains of the pathogens *Fusarium oxysporum* and *Verticillium dahliae*.

A total number of 21,99 regenerated plants resistant to *Fusarium oxysporum* f. sp. *lycopersici* and 9,66 plants resistant to *Verticillium dahliae* were obtained from the local variety Ideal at the highest concentration of pathogens (50%). From line L1620, 17,00 regenerated plants were obtained, showing resistance to a 50% concentration of the Fusarium culture filtrate, and 22,66 plants were resistant to *Verticillium dahliae.*

Keywords: Solanum lycopersicum L.; culture filtrate; somaclonal variation; explants; fungal pathogens; *in vitro* screening

Introduction

Tomatoes (*Solanum lycopersicum* L.) are a valuable horticultural crop that is grown and consumed worldwide. Optimal production is hindered by several factors, among which *Verticillium dahliae* and *Fusarium oxysporum* are considered significant biological constraints in temperate production regions. Both Fusarium and Verticillium wilts cause leaf yellowing and discoloration of the water-conducting tissues of the plant.

Every year, tomato is afflicted with several pathogens, including Verticillium, which limit their production. The pathogen is challenging to mitigate due to its broad host range and global distribution, as well as its ability to persist in soil for years (Acharya et al., 2020). Verticillium wilt is a devastating soil-borne fungal disease affecting various plant species, including tomatoes, and represents a serious threat to agricultural productivity (Mazzotta et al., 2022). The Verticillium wilt is caused by colonization of plant xylem vessels of a pathogenic fungus, *Verticillium dahliae*. As a result, the vascular system of the plants is altered, the supply of water and nutrients is limited, and chlorosis, defoliation, and plant death occur (Fradin and Thomma, 2006). Therefore, the control of the *V. dahliae* pathogen is essential for reducing losses in horticultural crops. (Eck et al., 2006).

Fusarium wilt of tomato, caused by *Fusarium oxysporum* f sp. *Lycopersici* is another economically important disease that affects tomato crops and significantly decreases yield. The pathogen affects a wide variety of hosts across all age groups and has been reported in nearly all parts of the world. The primary symptoms include wilting, chlorosis, and necrosis, premature leaf drop, browning of the vascular system, stunting, and damping off. Symptoms are more distinct in older plants during the transition between the blossom and fruit maturation stages (Kumari et al., 2023). The pathogen spreads quickly under favorable conditions, such as high humidity and high plant density in plantations, as well as disease incidence in cultivated plants, thereby reducing the effectiveness of fungicides (He, 2007; Hernández-Hernández, 2011).

Large-scale applications of chemical fungicides are commonly used to combat diseases. Excessive usage of these chemicals, on the other hand, always creates a risk of environmental contamination and poses a threat to all living forms by entering the food chain. The excessive use of chemicals is not only eventually harmful to human health but can also lead to resistance in pathogens that may be periodically exposed to these compounds (He, 2007; Ramírez-Mosqueda et al., 2019). Such problems negatively affect the yield and quality of tomato production and could be solved by the development of new high-yielding varieties that combine disease resistance and fruit quality, suitable for growing in different climatic conditions (Ivanova et al., 2023). In conventional breeding programs, various methodological approaches are employed to create sources of resistance against phytopathogens and extreme climate conditions. Traditional breeding methods often require time and yield genotypes with a significant number of heterogeneous genetic components. The integration of biotechnological techniques, such as somaclonal variation, into breeding programs may provide powerful tools for revealing genetic diversity and developing new varieties with disease resistance. When appropriate selection pressure is applied through a selection agent, plant organs, tissues, or cells that survive the selection pressure are potential sources of tolerant subclones (Rao and Sandhya, 2016). *In vitro* selection can be used to select disease-resistant plants using toxic culture filtrates or purified toxins as a selection agent (Sharma et al., 2010; Flores et al., 2012; Mahlanza et al., 2013). With this approach, the screening of a large number of plants in a limited space over a short period is possible (Patade et al., 2008; Suprasanna et al., 2009). Fungal metabolites present in culture filtrates can produce disease-like symptoms and trigger the elicitation of defense responses (Rao and Sandhya, 2016).

The use of laboratory methods in *in vitro* culture, which allows for the selection of cell populations resistant to specific factors and the subsequent regeneration of whole plants, yields high efficiency in assessing tomato genotypes for resistance to disease and various extreme environmental factors (Ivchenko et al., 2021). As a result of somaclonal variation in *in vitro* cultures, cell variants are formed that differ from original forms in important characteristics such as productivity and fruit size, varying levels of resistance to abiotic factors like salt tolerance (El-Sayed et al., 2004; Emilio et al., 2005; Hassan et al., 2008), and biotic stress (Švábová and Lebeda, 2005; García-Gonzáles et al., 2010; Srinivas et al., 2019). Resistant genotypes can be selected at any stage of their development, such as cells, callus, organogenic structures, or whole plants, thereby reducing the number of selection cycles required in conventional breeding programs (Ravikumar et al., 2007; Lebeda and Švábová, 2010).

The present study aimed to develop resistance in tomato plants against the soil-borne fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahliae* through an *in vitro* cell line selection approach.

Materials and Methods

Plant material

The experiments were carried out at Maritsa Vegetable Crops Research Institute, Plovdiv, with two tomato genotypes that showed good regeneration activity (established by previous experiments) – the Ideal variety and the L1620 line. The Ideal variety is a local cultivar with an indeterminate growth habit and an orange-red fruit color, susceptible to Fusarium and Verticillium wilts. Line L1620 is a somaclonal variant, obtained *in vitro*, with a determinate growth habit and a red fruit color.

***In vitro* conditions**

Seeds of tomato genotypes were surface-sterilized in a 5% calcium hypochlorite solution for 1 hour and then rinsed three times with sterile dH_2O. After that, the seeds were sown on basal medium containing macro- and microsalts by Murashige and Skoog, (1962), vitamins by Gamborg et al., (1968), 3% sucrose, and 0,7% agar for germination (MS), pH of the medium was adjusted to 5,8 with 0,1 M NaOH and/or 0,1 M HCl before autoclaving. Cotyledon explants (0,5 cm^2) were separated from *in vitro* grown plants, between the 7^{th} and 10^{th} day after seeding, and placed with the adaxial part up (downside of the cotyledons was touching the medium). The explants were cultivated on an induction medium supplemented with 2,2 mgL^{-1} 6-Benzylaminopurine (BAP) and 1,6 mgL^{-1} Indole-3-acetic acid (IAA). Three weeks later, the explants were transferred to culture medium MS supplemented with 2.2 mg L-1 kinetin and 1.6 mg L^{-1} IAA. To the medium were added three concentrations – 30% v/v, 40%

v/v, and 50% v/v of the culture filtrates of two strains of the pathogens *Fusarium oxysporum* (F1 and F2 strains) and *Verticillium dahliae* (V1 and V2 strains). The control treatment (0%) was exclusively MS medium without any culture filtrate. The Petri dishes with explants were incubated in a growth chamber at a temperature of 25 °C ± 1 °C, with a photosynthetic photon flux density (PPFD) of 200 µmol m^{-2} s^{-1}, a 16/8 h photoperiod, and subcultured at intervals of 20 days on the same medium variants. The experiment was carried out in three replications, with 20 explants for each of the different genotypes and medium variants. The callogenesis, organogenesis, regeneration frequency (percentage of explants with regeneration), and number of regenerants per explant were examined over 90 days.

Preparation of culture filtrates (CF)

The Verticillium and Fusarium isolates were part of the phytopathology collection at the Maritsa Vegetable Crops Research Institute in Plovdiv, Bulgaria. Filtrate preparation: Nine-centimeter-diameter Petri dishes containing potato-dextrose agar (PDA) were inoculated with isolates from *Fusarium oxysporum* and *Verticillium dahliae*. In the middle of the Petri dishes, a mycelial block was put from a 14-day-old culture of Verticillium and Fusarium and incubated in the dark at a temperature of 26 °C for 15 days. After 15 days, mycelium was collected from the fungus growth and diluted to a concentration of 2×10^6, and conidia were transferred to a liquid medium. The cultures are incubated in darkness at 26 °C. After 20 days, the cultures are filtered twice through filter paper to remove the fungal mycelium. The filtrate acidity was pH 5,7. The cultures are then filtered through syringe filters with pore sizes of 0.22 µm – 0.45 µm. Filtrates are added to the autoclaved and modified Murashige and Skoog (MS) medium with concentrations of 4×10^6.

Data analysis

The experimental data are presented as mean values and variance analysis and have been statistically processed using Microsoft Excel's Analysis tools. The difference between the control and treatment groups on the nutrient medium, expressed as a percentage (D%), has been calculated.

Results and Discussion

Different concentrations of culture filtrate (30%, 40%, and 50%) of the two strains of both pathogens (F1, F2, V1, and V2) were added to culture medium MS supplemented with 2,2 mgL^{-1} BAP and 1,6 mgL^{-1} IAA to induce shoot induction from cotyledon explants of two tomato genotypes. The results showed callus induction after 2 weeks of culture in all of the tested media. In both tomato cultivars, the cotyledon explants showed thickening and an increase in size after being placed on a nutrient medium. After 12-14 days, dense light green callus tissue begins to form initially at the margins of the explants, which subsequently expands and covers the entire explants (Figure 1).

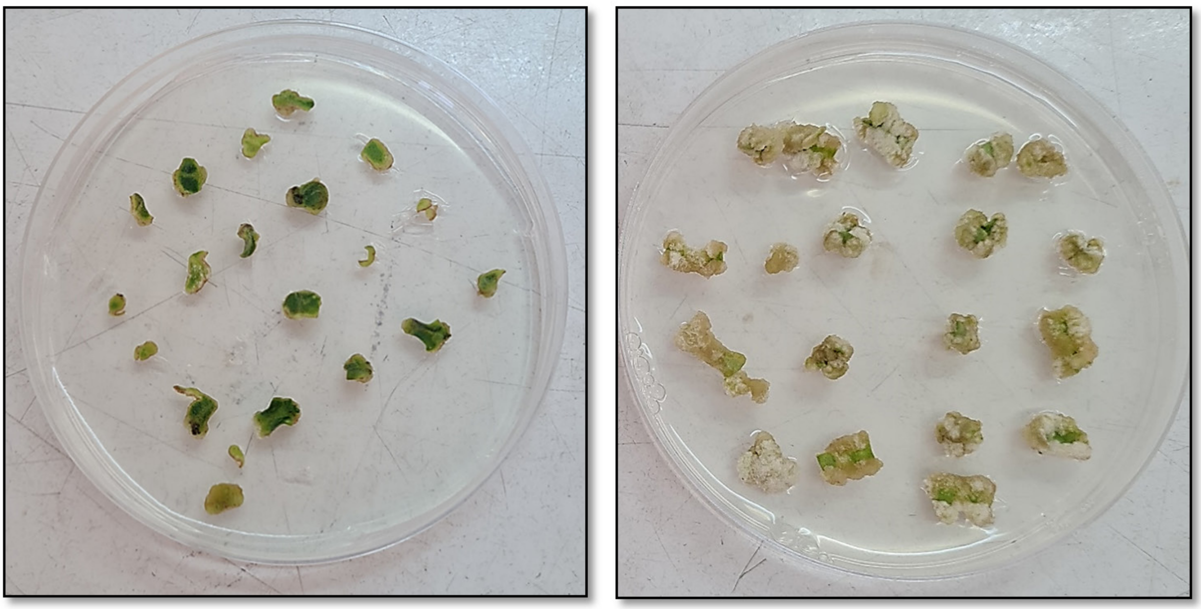

Fig. 1. Callused cotyledon explants of tomato plants

Organogenic structures are established in both investigated genotypes and across all treatment variants of the nutrient medium, ranging from 60% to 100% in the control and from 23.33% to 91.67% in those with added culture filtrates (Table 1). The best organogenic response (100%) was observed in the local genotype Ideal on the control nutrient medium. Explants showing the highest sensitivity to pathogen toxin filtrates begin to turn brownish and are unable to form organogenic structures, a phenomenon also reported by other authors (Storti et al., 1992) (Figure 2).

The inhibitory effect of pathogen culture filtrate exerts the most significant impact on the organogenic activity of the local variety Ideal, where even at the lowest concentration of culture filtrate (30%), a decrease in the number of organogenic explants ranging from -26,67% (V1) to -58,33% (F2) is observed. In contrast, the control variant in line L1620 exhibits lower organogenic activity (60%) than all concentrations and strains of *Fusarium* and *Verticillium* culture filtrates. In this case, it can be said that the pathogen culture filtrate exerts a stimulating effect on the specific genotype.

Ramírez-Mosqueda et al. (2015) establish that a 30% concentration of *Fusarium oxysporum* culture filtrate reduces the population of surviving regenerants by 50%. A similar trend has been observed by other authors (Ivchenko et al., 2021). The present study also shows a similar dependency – at a 30% concentration of *Fusarium* culture filtrate, the regenerative activity of the Ideal variety decreased from -65% to -66.10% (Table 2). In contrast, an almost identical effect was observed in organogenesis in line L1620, where the pathogen culture filtrate had a stimulating effect on regeneration, increasing from 80.02% to 95.02%. Similar results were obtained with a 30% concentration of Verticillium culture filtrates, where the number of regenerated explants in the Ideal variety was reduced by -40% to -60% compared

Table 1. Organogenic response of two tomato genotypes, following treatment with different concentrations of culture filtrates from the pathogens *Fusarium oxysporum* and *Verticillium dahliae*

Organogenesis								
Genotype	Control $\bar{x}$	Culture filtrate	30% $\bar{x}$	D%	40% $\bar{x}$	D%	50% $\bar{x}$	D%
L1620	60,00	F1	81,67	36,11ns	78,33	30,55ns	78,33	30,55ns
		F2	81,70	36,11ns	86,70	44,50*	83,33	38,88ns
		V1	91,67	52,78*	88,33	47,22*	80,00	33,33ns
		V2	76,67	27,78ns	91,66	52,77ns	90,00	50,00*
Ideal	100	F1	43,33	-56,67**	40,00	-60,00**	30,00	-70,00ns
		F2	41,67	-58,33**	61,66	-38,34**	61,66	-38,34**
		V1	73,33	-26,67**	40,00	-60,00**	36,66	-63,34**
		V2	53,33	-46,67***	53,33	-46,67*	23,33	-76,67***

C – control, D – difference, ns – non-significant; * – p≤0.05; ** – p≤0.01

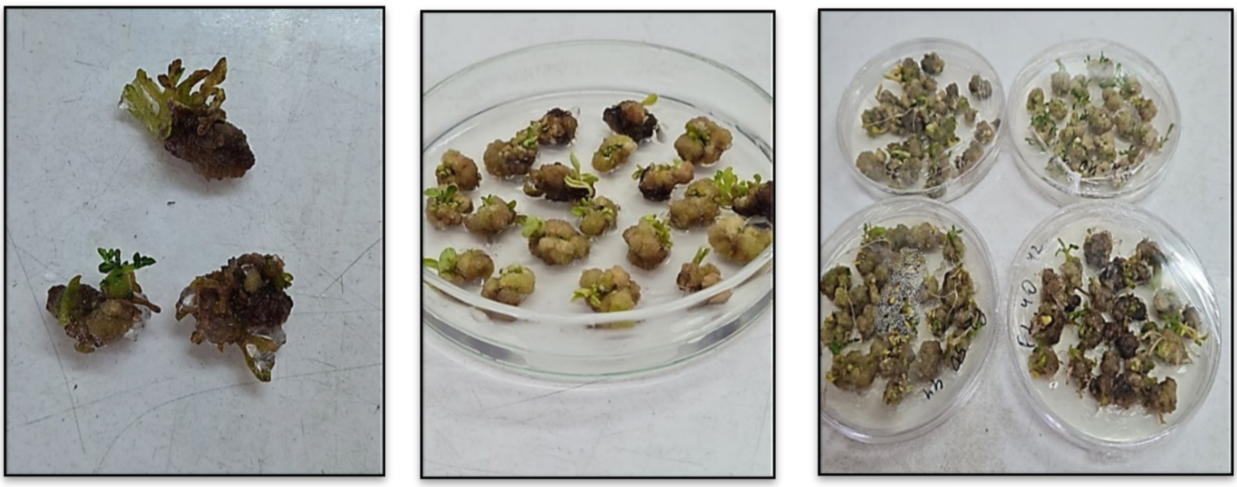

Fig. 2. Browning of sensitive explants under the influence of pathogen toxins

to the control variant. Once again, a stimulating effect on regeneration was observed in line L1620. With increasing concentrations of Fusarium and Verticillium culture filtrates, the regenerative potential in the genotype Ideal decreased (-71,67% to -85,00%) (Figure 3). An exception to this trend is the culture filtrate F2, where the number of regenerated explants increased to 51,66% at a concentration of 50%.

The experiments of Frame et al. (1991) demonstrated that a 20% culture filtrate of *Verticillium dahliae* reduced the number of regenerated plants by 50% compared to the control variant. The results of the present study show a similar tendency in the Ideal variety, where the number of regenerated explants decreased from 88.20% to 93.99% at a 30% filtrate concentration compared to the control variant (Table 3). On the other hand, a contrary trend was observed in line L1620, where an increase from 36,36% to 172,73% was registered, although the results were not statistically significant. A similar tendency was observed by other researchers, who found that the regeneration frequency also increased with an increase in the culture filtrate concentration of the pathogen *Cercosporidium personatum* (Venkatachalam et al., 1998). Similarly, Yu et al. (1990) obtained an increased frequency of callus on a medium containing *Verticillium albo-atrum* culture filtrates in alfalfa. Lynch et al. (1991) also described that the culture filtrate of *Alternaria solani* stimulated shoot bud regeneration in potatoes.

According to the studies by Ramírez-Mosqueda et al. (2019), the number of regenerated plants decreases with in-

Table 2. Regenerative response of two tomato genotypes following treatment with different concentrations of culture filtrate from the pathogens *Fusarium oxysporum* and *Verticillium dahliae*

Regeneration									
Genotype	Control $\bar{x}$	Culture filtrate	30% $\bar{x}$	D%	40% $\bar{x}$	D%	50% $\bar{x}$	D%
L1620	33,33	F1	65,00	95,02*	63,33	90,01ns	63,33	90,01ns
		F2	60,00	80,02*	66,66	100,00ns	80,00	140,02ns
		V1	86,66	160,01**	81,66	145,00**	75,00	125,02*
		V2	71,66	115,00ns	80,00	140,02**	76,66	130,00***
Ideal	100	F1	33,33	-66,10ns	33,33	-66,10ns	25,00	-74,58***
		F2	35,00	-65,00ns	45,00	-55,00**	51,66	-48,34*
		V1	60,00	-40,00**	30,00	-70,00*	28,33	-71,67*
		V2	40,00	-60,00**	48,33	-51,67*	15,00	-85,00*

C – control, D – difference, ns – non-significant; * – p≤0.05; ** – p≤0.01

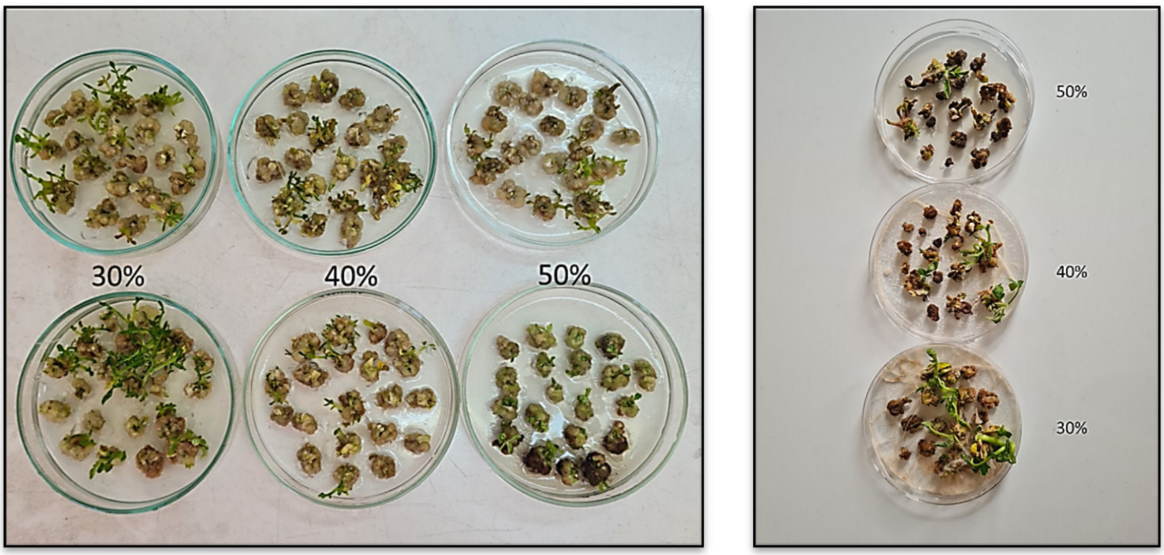

Fig. 3. Inhibitory effect of different concentrations of *F. oxysporum* f. sp. *lycopersici* culture filtrate on tomato genotype Ideal

Table 3. Number of regenerants per explant of two tomato genotypes following treatment with different concentrations of culture filtrate from the pathogens *Fusarium oxysporum* and *Verticillium dahliae*

Number of regenerants per explant								
Genotype	Control $\bar{x}$	Culture filtrate	30% $\bar{x}$	D%	40% $\bar{x}$	D%	50% $\bar{x}$	D%
L1620	0,33	F1	0,56	69,70ns	0,9	172,73ns	0,4	21,21ns
		F2	0,45	36,36ns	0,45	36,36ns	0,45	36,36ns
		V1	0,9	172,73ns	0,53	60,61ns	0,5	51,52ns
		V2	0,61	84,85ns	0,36	9,09ns	0,63	90,91*
Ideal	4,66	F1	0,28	-93,99**	0,45	-90,34**	0,48	-89,70**
		F2	0,4	-91,42**	0,3	-93,56**	0,61	-86,91**
		V1	0,5	-89,27**	0,28	-93,99**	0,31	-93,35**
		V2	0,55	-88,20**	0,58	-87,55**	0,16	-96,57**

C – control, D – difference, ns – non-significant; * – $p \leq 0.05$; ** – $p \leq 0.01$

creasing concentrations of pathogen filtrates. The results of the current experiment reveal a distinct trend, varying according to the specific strain of the pathogen and the corresponding genotype. In some cases, the tendency of reducing regenerated plants with increasing culture filtrate concentration is supported, as seen in line L1620 with the V1 strains, where the highest number of plants (18,00) was obtained at a concentration of 30%, and with a concentration of 40%, it was reduced to 12,33, and with a concentration of 50%, it was only 10,00 (Table 4). However, with the F2 culture filtrate, the number of plants remained unchanged (9,00) regardless of the concentration of the pathogen toxin. In the Ideal variety, on the other hand, an opposite trend was observed with the F1 filtrate: with an increase in filtrate concentration, the number of plants increased from 5.66 at a concentration of 30% to 10.33 at a concentration of 40% and 9.66 at a concentration of 50%.

In the scientific literature, *in vitro* selection of plants resistant to diseases caused by various pathogens has been reported for different plant species, including banana (*F. oxysporum* f. sp. *cubense*) (Hu et al., 2013), turmeric (*F. oxysporum* f. sp. *zingiberi*) (Kuanar et al., 2014), sugarcane (*F. sacchari*) (Mahlanza et al., 2013), pea (*F. solani*) (Švábová et al., 2011), etc. Most of these studies are based on somaclonal variation that occurs during the *in vitro* culture of plant tissues. However, some factors increase this variation through different mechanisms, including chemical mutagenesis (Purwati et al., 2007) or physical mutagenesis (Sharma et al., 2010). When conducting experiments with different concentrations of Fusarium filtrate, some authors believe that the filtration obtained *in vitro* from plants at a concentration of 50% (v/v) is of most significant interest for breeding (Ramírez-Mosqueda et al., 2019). However, Kuanar et al. (2014) obtained resistant plants of turmeric at a concentration of 7% of the filtrate, and Flores et al. (2012) obtained resistant plants of passionflower at a concentration of 14%.

In most studies, authors report a relationship between *in vivo* and *in vitro* resistance of plants selected (Mahlanza et al., 2013; Hu et al., 2013; Koyyappurat et al., 2015). A good correlation exists between *in vitro* screening and field responses on known cultivars, making these methods available for potential use as early screening tools (Predieri, 2001).

Table 4. Number of plants obtained from two tomato genotypes following treatment with different concentrations of culture filtrate from the pathogens *Fusarium oxysporum* and *Verticillium dahliae*

Number of plants obtained								
Genotype	Control $\bar{x}$	Culture filtrate	30% $\bar{x}$	D%	40% $\bar{x}$	D%	50% $\bar{x}$	D%
L1620	6,66	F1	11,33	70,12ns	18,00	170,27ns	8	20,12ns
		F2	9,00	35,14ns	9,00	35,14ns	9	35,14ns
		V1	18,00	170,27ns	12,33	85,14ns	10	50,15ns
		V2	12,33	85,14ns	7,33	10,06ns	12,66	90,09*
Ideal	93,33	F1	5,66	-93,94**	10,33	-88,93*	9,66	-89,65*
		F2	8,00	-91,43**	6,00	-93,57**	12,33	-86,79**
		V1	10,00	-89,29**	5,66	-93,94**	6,33	-93,22**
		V2	11,00	-88,21*	11,66	-87,51**	3,33	-96,43**

C – control, D – difference, ns – non-significant; * – $p \leq 0.05$; ** – $p \leq 0.01$

When tested with Fusarium filtration in *in vitro* conditions, Sowik et al. (2008) establish consistency in plant resistance *in vivo*, demonstrating the reliability of the *in vitro* method for selecting plant resistance.

Conclusion

As a result of the present experiments, somaclonal variants of tomatoes resistant to *Fusarium oxysporum* and *Verticillium dahliae* were obtained through *in vitro* culture and subsequent selection. The inhibitory effect of the culture filtrate on the ability of the genotypes to form regenerated plants was reported. A decrease in the regeneration activity was observed with an increase in the concentration of the culture filtrate of the pathogen in the Ideal variety. While in line L1620, the opposite trend was observed – the most significant number of regenerated plants was obtained on the media with added filtrates of the pathogens, compared to the control variant. In this case, the toxins of the pathogen exerted a stimulating effect.

A higher number of regenerated plants (22.66) resistant to Verticillium culture filtrate at 50% was obtained from line L1620, and a total of 17.00 plants were resistant to Fusarium. The plants obtained from the local variety Ideal, resistant to the highest concentration of Fusarium culture filtrate (50%), were 21,99, and 9,66 regenerated plants were resistant to Verticillium culture filtrate (50%). The obtained results may contribute to a possible solution for controlling soil-borne pathogens in tomatoes and provide a basis for further genetic and biotechnological research on tomatoes.

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