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In vitro screening for herbicide selectivity of new mutant pepper genotypes with different origin and fruit colour

In vitro скрининг за хербицидна селективност на нови мутантни генотипове пипер с различен произход и оцветяване на плода

Svetla YANCHEVA[1] (✉), Nikolay PANAYOTOV[1], Nasya TOMLEKOVA[2]

[1] Agricultural University Plovdiv, 12, Mendeleev Blvd., 4000 Plovdiv, Bulgaria

[2] Maritsa Vegetable Crops Research Institute, 32, Brezovsko shosse Str., 4003 Plovdiv, Bulgaria

✉ Corresponding author: svetla20@hotmail.com

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ABSTRACT

The study presents an *in vitro* test development as a model for herbicide phytotoxicity. It provides reliable data on how the herbicide affects the seed germination and early growth stages, in dynamic, during the cultivation. The sensitivity of five mutant pepper genotypes with different origin and fruit colour to the herbicide napromamide (Devrinol 4F) was investigated. All studied herbicide doses, corresponding to 3, 4, and 5 L/ha, caused phytotoxicity expressed by decreased germination and growth inhibition. A strong genotype dependence was established – two breeding lines (18 and 85), originating from local populations, demonstrated a low sensitivity to the herbicide, additionally confirmed by higher plant survival and adaptability after transplanting to soil *ex vitro*.

Keywords: *in vitro*, local populations, mutants, napromamide, pepper, phytotoxicity

РЕЗЮМЕ

Това проучване представя разработването на *in vitro* протокол като модел за хербицидна фитотоксичност. Чрез него се осигуряват надеждни данни за това как хербицидът влияе на кълняемостта и ранните фази на растеж, в динамика по време на култивирането. Изследвана е чувствителността на пет новоселектирани генотипи пипер с различен произход и цвят на плода към хербицида напромамид (Devrinol 4F). Всички приложени дози на хербицида, преизчислени към 3, 4 и 5 L/ha, причиняват фитотоксичност, която се проявява чрез намаляване кълняемостта на семената и инхибиране на растежа. Установена е силна генотипна зависимост - две от линиите (18 и 85), произхождащи от местни популации, демонстрират по-ниска чувствителност към хербицида, която допълнително се потвърждава и чрез по-високата преживяемост на растенията и адаптивност след засаждане в почва *ex vitro*.

Ключови думи: *ин витро*, местни популации, мутанти, напромамид, пипер, фитотоксичност

INTRODUCTION

Pepper growing and the development of new varieties have a long-standing tradition in Bulgaria. The main challenges in pepper breeding are directly related to the cultivation conditions, soil and climatic conditions, applied agro-technology, resistance to pesticides controlling the development of diseases, pests, and weeds, and the cultivars' improvement towards high nutritional value.

Peppers do not tolerate weed competition. Therefore, the studies are directed to different agrotechnological approaches such as mulching, hand weeding, hoeing the soil around pepper plants to eliminate the weeds, and chemical control. Despite different application timing, most of the studied herbicide formulations caused phytotoxicity expressed by chlorosis, inhibited and abnormal growth. Moreover, fresh and processed pepper consumption requires precise monitoring of the residues counting selectivity and phytotoxicity.

Several herbicides have been reported as selective for pepper, but research supporting their use for weed control in this crop is limited (Figueroa et al., 2016). Chemical control of weeds is still topical and disputable, and the reports concerning herbicide selectivity in pepper demonstrate the attempts to find efficient formulas with the absence or low level of phytotoxicity expressed in different stages of plant development.

A wide range of formulas based on various active substances has been tested both pre- and post-plant or combined with agrotechnological practices. Field and greenhouse comparative experiments found that diphenamide has a lower toxic effect than the use of napropamide (Eshel et al., 1973). The result of mulching and herbicide co-administration in pepper seedlings was investigated by Cavedo et al. (1996), pointing out that napropamide and diphenamide are selective. However, napropamide does not affect deciduous weeds, while clomazone caused phytotoxicity expressed by chlorotic spots. In field-grown peppers, Gilreath et al. (2004) reported that the best weed control was obtained by applying high doses and deeper incorporation of napropamide.

The efficiency of clomazone and trifluralin in peppers was described by Masabni et al. (2013). Clomazone, S-metolachlor, and pendimethalin used alone or combined after transplanting demonstrated a good selectivity and tolerance (Mohseni-Moghadam and Doohan, 2015a; Figueroa et al., 2016; Glatkova and Pacanoski, 2019).

Peppers demonstrated sensitivity to sulfonylurea-based herbicides. As a result, the overall plant development was suppressed, the number of flowers and fruits decreased, finally resulting in reduced yield (Pekarek et al., 2013). Evaluation of halosulfuron, imazosulfuron, and trifloxysulfuron applied through a drip irrigation system revealed a better tolerance in field-grown peppers than under greenhouse conditions (Dittmar et al., 2016).

Low selectivity was also reported to paraquat and mix with flumioxazin, and the observed tolerance was better in a single application (Natan, 2014). Field experiments with 2,4-D, Dicamba, and 2,4-D plus glyphosate demonstrated pepper sensitivity even to low doses of both preparations (Mohseni-Moghadam and Doohan, 2015b). It was concluded that pepper yield depends significantly on proper spacing and proper use of herbicides, suppressing weeds and increasing the yield (Mustapha et al., 2020).

The existing genetic diversity in peppers and the enriched assortment in the breeding collections of pepper varieties, accessions, and lines are the base for successful and focused breeding activities. More often, the researchers use many local populations and varieties, including them in hybridization programs. Based on stabilized hybrids obtained up to 6-10 generations, they realize productive varieties with high yield, good plasticity, and adaptability, and high nutritional value (Ambarus et al., 2010; Cvikić et al., 2011; Li et al., 2012; Zhang et al., 2013; Martirosyan and Sargsyan, 2014; Morton, 2014; Nankar et al., 2020).

The present targeted *in vitro* study was performed to evaluate the sensitivity of newly developed pepper genotypes to napropamide (Devrinol) as a widely used herbicide in this crop and the post-treatment adaptability of the plants.

MATERIALS AND METHODS

Plant material

Seeds were obtained from pepper plants grown at the Agricultural University in Plovdiv and the Maritsa Vegetable Crops Research Institute in Plovdiv (Bulgaria). Based on conventional and mutation breeding methods aiming to create genetic diversity, the selected lines have been developed and presented as valuable new genotypes with different fruit colour and high nutritional value. The origin and description of the studied genotypes are shown in Table 1.

Sterilization procedure

Pepper seeds were examined for the absence of damages and treated with a solution of commercial bleach (4.5–5% of active chlorine) used at the final concentration of 10% (*v/v*) (0.45–0.5% active chlorine) for 10 min and finally rinsed three times for 5 minutes with sterile distilled water in a laminar box. Seeds were blotted dry by sterile filter paper, and five seeds were introduced in a cultivation container. Small glass jars (180 mL) containing 30 mL medium were used as culture vessels.

Herbicide treatment

Devrinol 4F is a selective soil systemic herbicide with the active substance napropamide (450 g/L) prepared as a suspension concentrate. The manufacturer's recommended dose is 4 L/ha. Its phytotoxicity on the selected pepper lines was studied *in vitro* by the following treatments: 3, 4 (recommended), and 5 L/ha compared to control (untreated).

The cultivation vessel area was determined by the formula $S = \pi . r^2$ and herbicide stock solutions with appropriate concentrations were prepared. The herbicidal solution (0.5 mL) was applied as a thin layer on the solid culture medium surface by a pipette under sterile conditions. Each treatment (herbicide dose) and control consisted of five vessels with five seeds.

Cultivation

The nutrition medium in all experiments was a modified basal medium MS (Murashige and Scoog, 1962), with a reduced concentration of macronutrients (½ MS), enriched with sucrose (20 g/L), agar (7 g/L), and pH = 5.7 prior autoclaving. Plant development was performed in a

Table 1. Origin and description of the studied genotypes

Pepper genotypes	Mutant lines		Hybrids obtained by a transfer of induced mutations into local varieties		Hybrid breeding line
	18-2010-8/5 (Line 18)	85-1/4–2008 (Line 85)	145–2011/1 (Line 145)	147–2011/2 (Line 147)	34–2006/10–4 (Line 34)
Origin	Spontaneous mutation in the local variety Osmarsko kambe	Spontaneous mutation in a local population from Svishtov region	Hebar x M_8 Advanced Mutant Line 1930	Kurtovska kapia 1619 x M_8 Advanced Mutant Line 1930	Doux Marconi San Semences x Kalinkov 807/5
Fruit colour	Orange	Orange	Orange-red	Intensive orange	Dark red
Shape	Round type	Conic	Conic	Conic with slight longitudinal wrinkles	Elongated block type, with 3-apexes
Weight (g)	47.4±1.02	76.0±1.21	84.0±1.42	102.0±2.33	119.0±2.86
Length (cm)	4.7±0.26	12.2±0.61	14.2±0.66	15.4±0.73	15.8±0.87
Diameter (cm)	4.6±0.41	4.2±0.48	3.8±0.26	4.6±0.38	6.3±0.78
Pericarp thickness (mm)	5.0±0.13	4.5±0.22	4.5±0.0.18	4.0±0.12	7.0±0.33
Taste	Hot	Sweet	Sweet	Sweet	Sweet

growth chamber with temperature 22±1 oC, 2500-3000 Lux light intensity, and 16/8 h photoperiod.

The herbicide effect was evaluated actively on days 7, 14, and 21 by reporting the following indicators as mean values: seed germination, plant height, root length, and number of lateral roots. After day 21, all developed plants or germinated seeds were potted to a peat-perlite (3:1) substrate and cultivated in a growth chamber with a gradual decrease in atmospheric humidity. After day 35, the survival and growth performance of the plants were observed. The results based on three independent experiments were subjected to statistical analysis.

RESULTS AND DISCUSSION

Effect of the herbicide on seed germination in vitro

According to the ISTA Rules, the seed germination was checked preliminary under standard laboratory test, and the values obtained were 98-100%.

Plant development in the early stages is essential for further growth. The results demonstrate the dynamic of seed germination *in vitro* and the herbicide action in all studied genotypes (Figure 1).

In the control variant (Fig.1A), 100% seed germination at day 7 was reported only in line 85. At the end of the cultivation (day 21), maximum seed germination was reached in genotype 18, while lines 34 and 147 had 75% germination. Line 145 demonstrated the lowest germination (35%), and it can be explained by the genotype specific characteristics and response *in vitro*. However, seed disinfection is a recommended procedure before the plantation of several crops, ensuring healthy plant growth. It could be suggested that chlorine's effect during seed sterilization could be an additional factor for decreased germination.

Herbicide application significantly influenced the germination and subsequent plant development, causing abnormal growth *in vitro*. In the napromamide treated variants (Fig.1B, C, D), a delay in germination and inhibited shoot and root development were observed. Lines 85 and 18 demonstrated better germination and growth rates, while all the other lines were significantly depressed. The best herbicide tolerance was established in line 85, with 100% germination and seed development (3 L/ha), 96% (4 L/ha), and 88% (5 L/ha). The most sensitive was line 145, with the lowest germination (also observed in control).

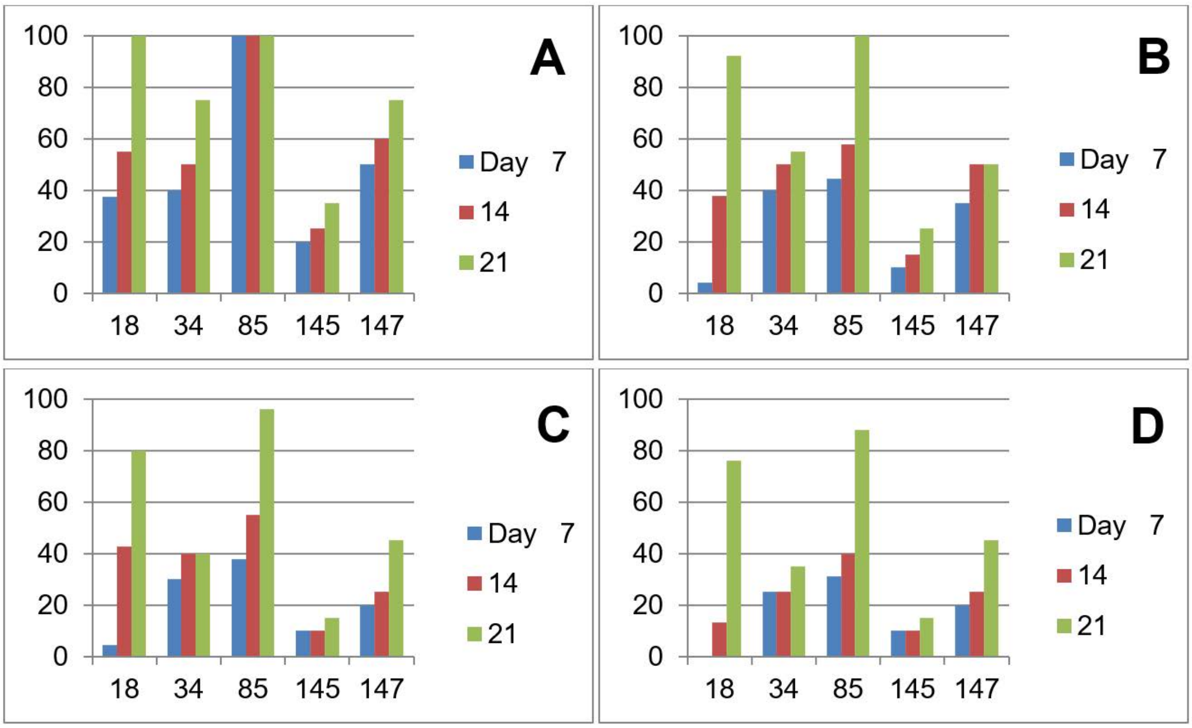

Figure 1. Seed germination in the control (A) and herbicide treatments of 3 L/ha (B), 4 L/ha (C), and 5 L/ha (D) *in vitro*

However, the herbicide inhibition percentage was comparable with the other lines. The later experimental events showed the complexity of the factors influencing the early stages of plant development.

Effect of the herbicide on plant development in vitro

The initial development of the root system in cultivated plants is critical for their survival and further growth. Figure 2A shows the reported data for the root length in control and treated variants.

Within the control, the most extended root (length) was reported in line 145 (1.4 cm) and line 85 (1.06 cm) on day 21. The root growth was influenced, i.e., as the concentration increased, the root length decreased.

In the herbicide treatments, the highest values were established in line 85 - 0.92 cm (3 L/ha), 0.67 cm (4 L/ha), and 0.64 cm (5 L/ha), respectively. The percentage of inhibition varied from 13 (3 L/ha) to 40 (5 L/ha). In the other lines, the root length decline was more substantial and genotype-dependent.

The number of lateral roots is an essential indicator for assessing herbicide impact and its phytotoxicity (Figure 2B). The most substantial reduction (up to ten times) in the number of lateral roots was demonstrated in line 145 where on day 21 the index decreased from 9.85 (control) to 1.5 (3 L/ha), 1.25 (4 L/ha), and 0.9 (5 L/ha) indicating genotype sensitivity. Comparable inhibition was also seen in lines 18 (up to 90%) and 85 (40-90%).

Data for plant height once more demonstrated the genotype- and dose-dependent effect of the herbicide (Figure 3). In lines 18, 85, and 145, an average of twice inhibited growth and development was noted, while in 34 and 147, it was still more significant.

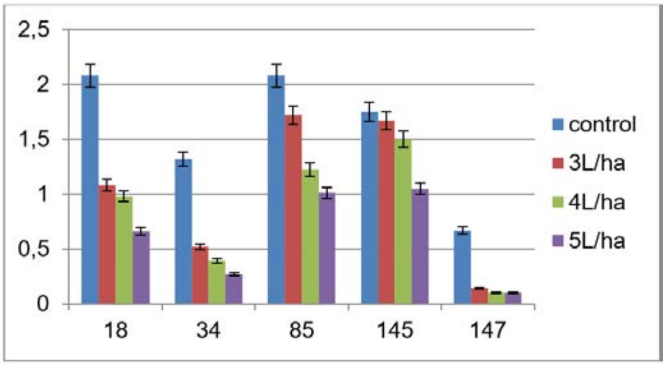

Figure 3. Effect of the herbicide concentration on plant height (cm) *in vitro* on day 21. Vertical bars show the standard error (±SE)

The characteristics that revealed phytotoxicity *in vitro* were: decreased and delayed germination and inhibited growth expressed by reduced root length, number of lateral roots, and plant height. Other distinctive symptoms were leaf and shoot malformations, root and shoot stunting, although leaf chlorosis (yellowing) and leaf necrosis (death) were not observed (Figure 4).

Ex vitro plant development

The established genotype sensitivity by the *in vitro* test was also confirmed in the subsequent in vivo plant development. The survival percentage was 100 only in genotypes 18 and 85 (control and herbicide pre-treated plants), (Figure 5).

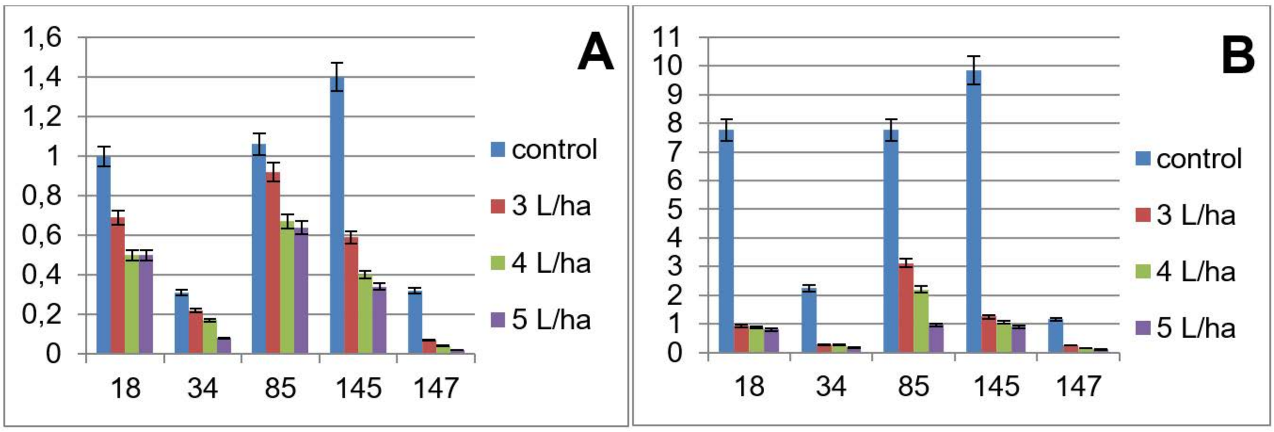

Figure 2. Effect of the herbicide concentration on (A) root length (cm) and (B) lateral root number *in vitro* on day 21. Vertical bars show the standard error (±SE)

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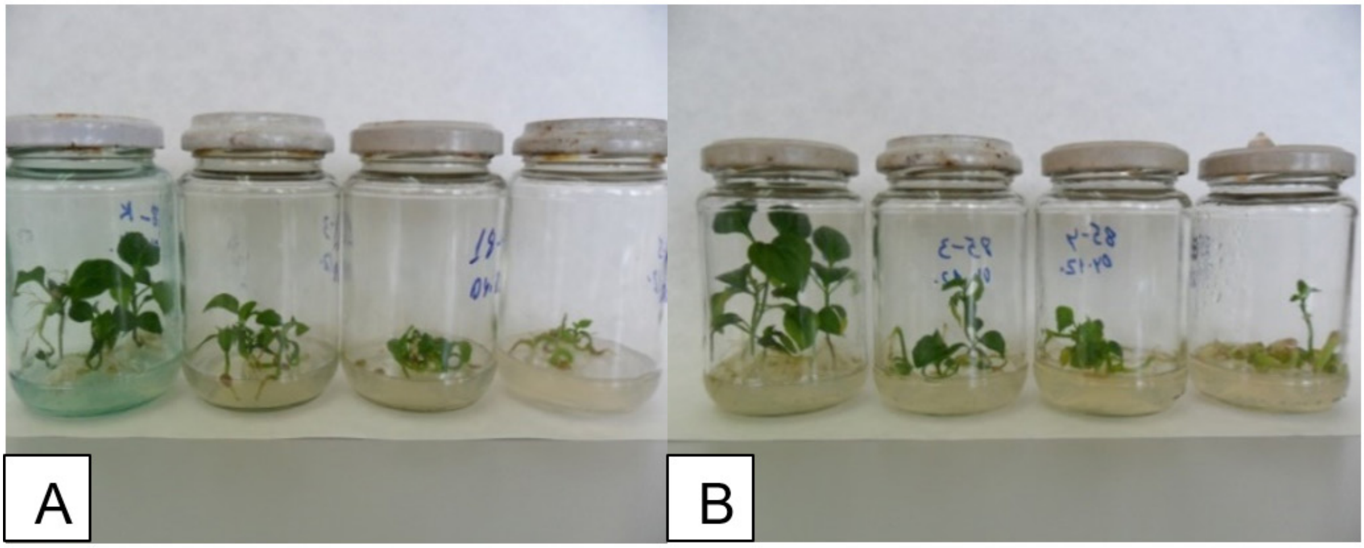

Figure 4. Plant development *in vitro* in the control and herbicide-treated variants of 3, 4, and 5 L/ha (left to right), (A) line 18, (B) line 85 at day 21

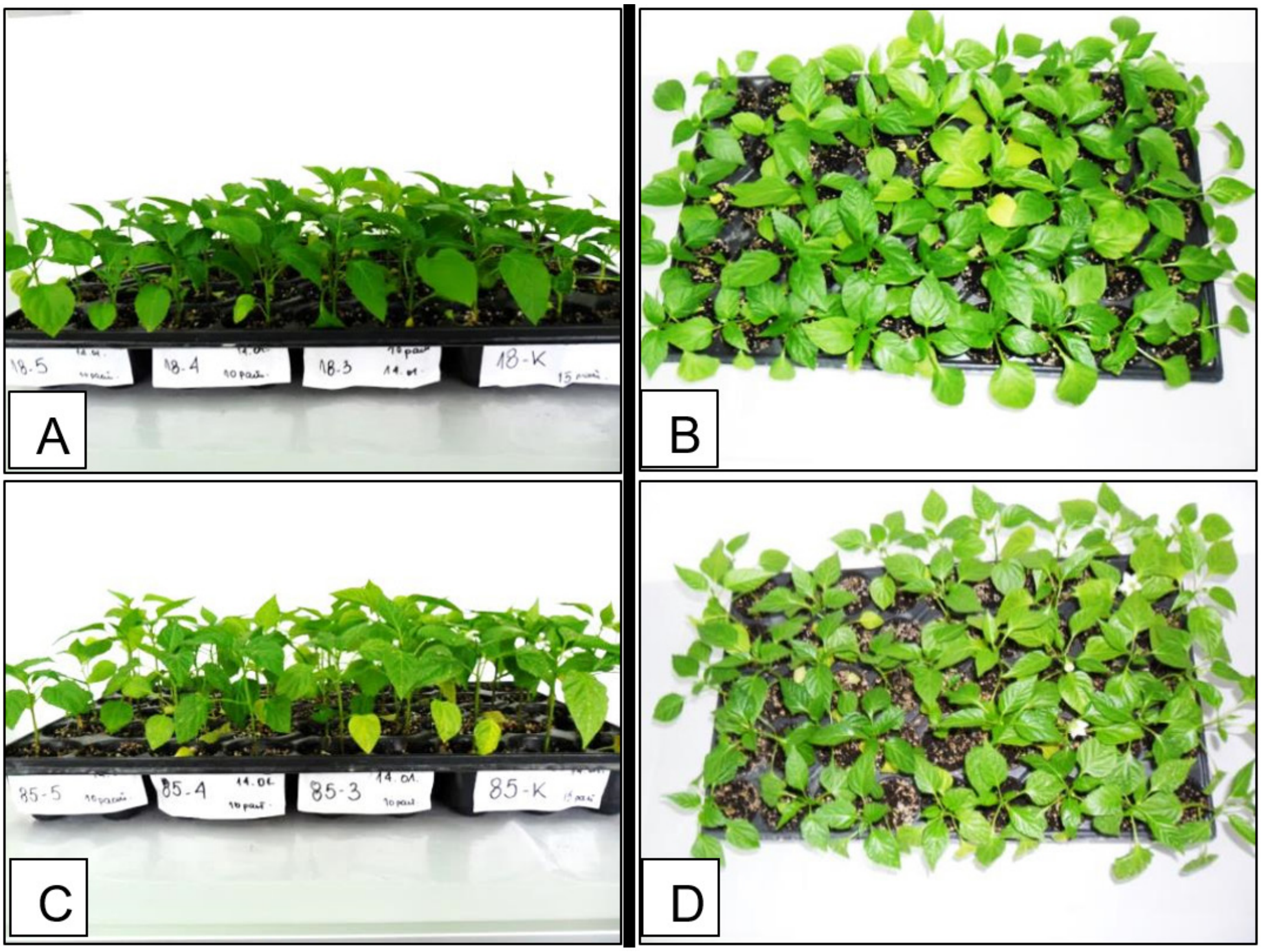

Figure 5. Plant development in vivo in the control and herbicide-treated variants of 3, 4, and 5 L/ha (right to left) (A, B) line 18, (C, D) line 85 at day 35

All other lines had a lower survival rate (20-70%) in control, while in the pre-treated variants it ranged from zero to 50 % depending on the herbicide dose. Nevertheless, all survival plants were measured at day 35, and the indexes, characterizing their growth as plant height and number of leaves (based on a minimum of five survived plants), are presented in Figure 6.

Observing the adaptability *ex vitro* and subsequent plant development, we found that in the plant height indicator (Figure 6A), only line 18 plants pre-treated with 3 L/ha demonstrated a higher value than the control. It could be suggested that the low dose or residual herbicide effect influenced stem elongation. Herbicide (3, 4, and 5 L/ha) caused growth retardation in all other genotypes. Similarly, the mean number of leaves (Figure 6B) in line 18 (treatment 3 L/ha) exceeded the control, while in line 85, it remained the same. At all other genotypes, the herbicide post-effect was still visible. Lines 147 and 34 were identified as the most sensitive. Only control and single pre-treated plants survived, also forming elongated stems.

The napromamide action, including blocked germination, absorption through the roots, reduced lateral roots and hypocotyl, could explain the established data and the observed growth behaviour. Additionally, for the entire period (21-days) of the *in vitro* cultivation, the seeds and the developed seedlings are continually in direct contact with the herbicide solution. It penetrated the seeds with the moisture of the culture medium, influencing cell division and further overall plant development and, in this way, causing additional stress. This effect was identified in all studied lines, but it was more noticeable in the more sensitive genotypes.

DiTomaso et al. (1988) reported that napropamide reduces the rate of entry of pea root cells into DNA synthesis and cell division by 8 and 12 hr of treatment, respectively. The inhibitory effect of napropamide on the mitotic cycle may result from an inhibition in the synthesis or activity of cell cycle-specific proteins.

The results from the present study are in agreement with some of the previously established herbicide effects in peppers. Andrew et al. (2000) stated that the norflurazon application was associated with inhibition of carotenoid biosynthesis in pepper plants and caused toxicity. According to Figueroa (2016), the pre-transplant applied herbicides bentazon, clomazone, napropamide, oxadiargyl, and oxadiazon produced intermediate phytotoxicity (higher than 25% foliar chlorosis). Comparing diphenamid and napropamide for phytotoxicity to pepper grown under glasshouse and field conditions, Eshel et al. (2006) revealed that napropamide caused retaining root and elongation shoot growth of seedlings. Plant growth was suppressed when the roots were exposed to the herbicides, but the shoots, through treated soil were not adversely affected.

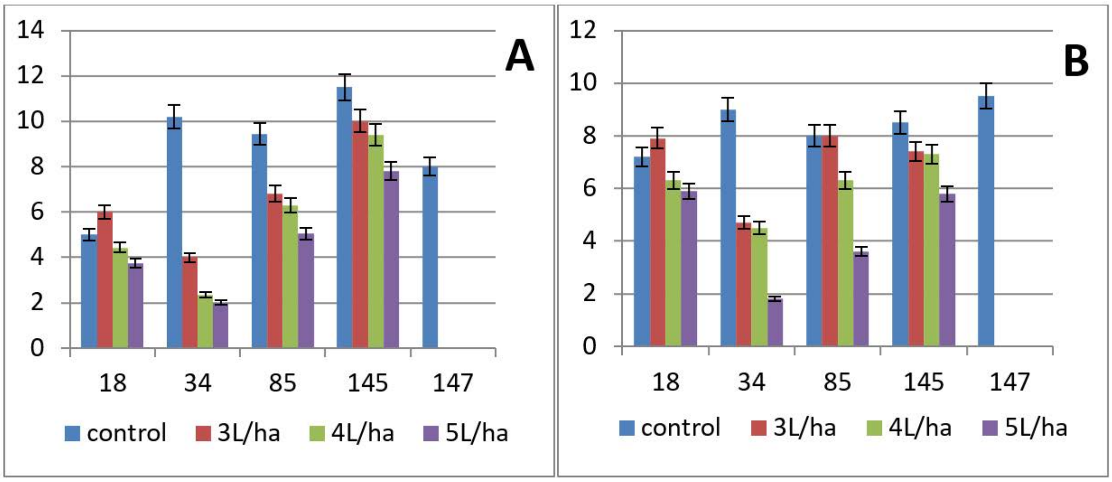

Figure 6. (A) Plant height (cm) and (B) number of leaves in the control and herbicide pre-treated variants (3, 4, and 5 L/ha) grown *ex vitro*

Recently, safeners have been extensively used to reduce phytotoxicity to crops, thus serving as an alternative weed control strategy. The use of safeners promotes greater crop selectivity, allowing the application of herbicides with different mechanisms of action on the crop (Castro et al., 2020).

The current study determined that the timing of herbicide application in the early stages of plant development is crucial for their further growth. The sensitive pepper genotypes do not recover and cannot overcome the effect of the herbicide. Lines 18 and 85, originating from local populations, demonstrated a better herbicide tolerance which was confirmed by their higher adaptability and survival rate after transplanting to the soil.

CONCLUSION

The present study was performed as a model for comparative evaluation of stabilized pepper breeding lines concerning their susceptibility to herbicide napromamide under controlled *in vitro* conditions. The assessment of the adaptive potential of the genotypes *ex vitro* gives additional knowledge for their growth behaviour and selection for further field experiments.

The developed *in vitro* screening protocol for herbicide selectivity is easy for performance, time-saving, and independent from environmental factors. Moreover, it has an economically and ecologically beneficial impact. It is a basis for recommending similar investigations providing preliminary information on the herbicide's phytotoxicity, especially for testing new varieties and breeding lines' susceptibility and new herbicide formulations.

Furthermore, the gained results support the view that modern breeding for stress resistance will be increasingly based on using local varieties and populations adapted to the specific soil and climatic conditions or to a specific eco-geographical area.

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