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IN VITRO STRESS SELECTION OF MARKER FREE TRANSGENIC POTATO LINES IN ORDER TO PRODUCE POTATO RESISTANT TO BOTH PVY AND DROUGHT STRESS

RALUCA-ALINA MUSTAŢĂ [1], IMOLA MOLNAR[1], TUNDE-EVA DENES[1] and ELENA RAKOSY-TICAN[1]✉

SUMMARY. Potato, an excellent staple food, is one of the main targets for genetic improvement by combinatorial biotechnology. The aim of the present study was to combine gene transfer with *in vitro* stress selection to obtain plants resistant to both PVY and drought stress. Our hypothesis is that one resistance to one type of stress might determine a state of better response to a second stress factor. Marker free, transgenic potato lines were tested for resistance to drought under *in vitro* polyethylene glycol (PEG) induced stress selection. Callus cultures have been obtained from internodes of potato cultivars: Baltica and Désirée, of control and transgenic lines, respectively. The drought stress was simulated *in vitro* for three weeks on MS-T medium with 5% PEG 6000. The regenerated plants were selected in a second round on RMB5 medium with 5%, 10% and 15% PEG. At the end of each stage, the plants were evaluated for viability, root development and the regeneration of axillary buds. Two clones of cv. Baltica putatively tolerant to drought and resistant to PVY were obtained.

Keywords: drought, PEG, potato stress selection.

Introduction

Potato (*Solanum tuberosum*) belongs to the Solanaceae family and is the third crop in the world after rice and wheat (Birch *et al*., 2012). It spreads to all continents and is cultivated in more than 150 countries, with varied uses in food, as animal feed and also in various industries (Muthoni *et al*., 2013). However the potato is very sensitive to diseases, pests and abiotic factors such as drought. Important losses in the annual potato production are also caused by pathogens or pests such as: Colorado potato beetle (*Leptinotarsa decemlineata*), late blight (*Phytophthora infestans*) and viruses (PVY, PVX, PRLV etc.).

[1] *Babeş-Bolyai University, Faculty of Biology and Geology, Plant Genetic Engineering Group, Clinicilor str., no. 5-7, Cluj-Napoca, Romania.*

✉ ***Corresponding author: Elena Rakosy-Tican**, Babeş-Bolyai University, Faculty of Biology and Geology, Plant Genetic Engineering Group,*
E-mail: arina5744@yahoo.com

Potato virus Y (PVY) is an economically important virus, which is transmitted by aphids and infects crop species in the Solanaceae family. PVY has a high genetic variability, a significant number of strains being described so far: PVY^{O}, PVY^{C}, PVY^{Z}, PVY^{N} (Tribodet, *et al*., 2005). The recently identified strains, PVY^{NTN} (N-tuber-necrosis) and PVY^{N-W} (N-Wilga), have a rapid spread causing severe yield reductions, up to 80%. Both PVY^{NTN} and PVY^{N-W} induce Potato Tuber Necrotic Ringspot Disease (PTNRD) (Blanco-Urgoiti *et al*., 1998; Visser *et al*., 2012). Knowing that sources of natural virus resistance in plant are limited, novel resistance sources using transgenesis have been developed. Some genetic transformation methods including protein and RNA-mediated approaches (Prins, 2003) or virus-induced gene silencing (Lu *et al*., 2003; Godge *et al*., 2007) were used to induce virus resistance in plants (Baulcombe, 1999). In other studies the virus resistance in susceptible plants has been induced by transferring virus-derived genes which are including viral coat protein (CP) (Reddy *et al*., 2010) or CP genes in an intron- hairpin construct (Yan *et al.*, 2009). In previous experiments transgenic marker-free potato was produced using a two-step protocol. In the first step the transfer and expression of reporter gene *gfp* (green fluorescent protein) and marker gene *npt*II (neomycin phosphotransferase) were used to improve transformation of different cultivars of potato (Rakosy-Tican *et al.*, 2006). In the second step *A. tumefaciens* C58C1 pGV2260 with the hairpin construct pRGG YCPiPCY (35SCaMV enhancer and promoter, two repeated inverted PVY-CP sequences separated by an intron and pAnos terminator) was used for transforming the best responding potato cvs, Baltica and Désirée to resist PVY (Rakosy-Tican *et al.*, 2010). Resulting transgenic lines of two cultivars Baltica and Désirée were used in the present study for *in vitro* drought stress selection. Water deficit is a frequent stress in potato production, causing lower quality and reduced tuber yield (Steduto *et al*., 2012). Potato is very sensitive to water stress because of its shallow root system (Ekanayake and Midmore, 1992; Dalla Costa *et al*., 1997). One biotechnological method to obtain drought-resistant plants is *in vitro* selection under water stress conditions. To select *in vitro* drought resistant plants polyethylene glycol (PEG) is used as a component added to the culture media (Hassanpanah, 2010; Pino *et al.*, 2013). The treatment with PEG 6000 applied to seven potato cultivars allowed the selection of one drought resistant cultivar (Hassanpanah, 2010). In another study the drought conditions were simulated *in vitro* by the addition of PEG 4000 in concentration of 0%, 4% and 8% added to MS medium (Murashige and Skoog). These authors used both *Solanum tuberosum* and *S. commersonii* transgenic lines which overexpress the gene *ScCBF1* and compared them with the wild type after *in vitro* drought stress. Three different concentrations of PEG reduced the viability, but the transgenic plantlets were 100% viable in both species (Pino *et al*., 2013).

In this study *in vitro* stress selection of transgenic potatoes integrating a hairpin construct, hence putatively resistant to PVY and of their wild type counterparts was applied with the aim to combine these two resistance traits. Plant viability and

root regeneration rate indicate that two transgenic clones of cv. Baltica do resist *in vitro* drought stress. These lines are going to be further analyzed and provide an example of combining different resistance traits by biotechnological means.

Materials and methods

Selection of drought resistant plants

In this study we used two potato cultivars: Désirée and Baltica, which have been previously transformed using *Agrobacterium tumefaciens*, C58C1 pGV2260 with the hairpin construct pRGG YCPiPCY (35SCaMV enhancer and promoter, two repeated inverted PVY-CP sequences separated by an intron and pAnos terminator), to obtain transgenic potato lines resistant to PVY. Putatively transgenic lines were identified by PCR amplification of the construct, using two different designed specific primers (Rakosy-Tican *et al*., 2007).

The wild type (wt) Désirée (http://www.europotato.org) and Baltica (Solana GmbH & Co K6, Germany), the transgenic lines of cv. Désirée coded 4D1, 6D4, 6D6 and putatively transgenic (not analyzed yet at molecular level) 1D6, 1D9 and 6D1, as well as transgenic lines of cv. Baltica 5B2, 5B8, putatively transgenic 1B4, 3B5, 4B9, 6B4, 6B8 and nontransgenic 7B3 were used in the experiments.

The selection of plants resistant to drought stress was performed in two rounds: at callus level and then on plantlets exposed to increasing concentrations of PEG. Callus was induced on internodes of cvs. Baltica and Désirée of both control and transgenic potato, cultivated on MS-T medium (MS-based medium with 16 g L^{-1} glucose, 0.5 mg L^{-1} folic acid, 0.05 mg L^{-1} biotin, 40 mg L^{-1} adenine, 0.02 mg L^{-1} GA3, 0.02 mg L^{-1} NAA, 2.0 mg L^{-1} zeatin riboside and 0.7 % (w/v) agar, at pH 5.8). The callus was cultured three weeks on MS-T medium with 5% PEG 6000. Then the callus with first regenerated shoots was transferred on MS-T medium, without PEG. Potato plants were maintained in a growth room at 21 ºC with a photoperiod of 16 h (90 µmol m^{-2} s^{-1}, daylight fluorescent illumination). Plants regenerated, after stress, were evaluated for viability and capability to regenerate new plants. In the second round of selection, the lines which regenerated plants from callus under stress conditions were transferred on RMB5 medium (Menczel *et al*., 1981) with 5%, 10% and 15% PEG, each applied for three weeks. The lines with viable plants were selected on RMB5 with 10% PEG, simulating the drought conditions for other three weeks. Finally, the lines with viable plants, after stress induced by 10% PEG, were tested, in the next step of selection, on RMB5 with 15% PEG for another three weeks. At the end of each step the tested plants were evaluated for viability and regeneration rate. The data were statistically analyzed using Microsoft Excel and R statistics software (*http://www.lsw.uniheidelberg.de/users/christlieb/teaching/UKStaSS10/R_refman.pdf*).

Results and discussion

Although plant selection efficiency on drought stress conditions was dependent on genotype, all tested genotypes successfully regenerated callus and further on, all genotypes regenerated plants under stress conditions i.e. when 5% PEG was added to the culture media.

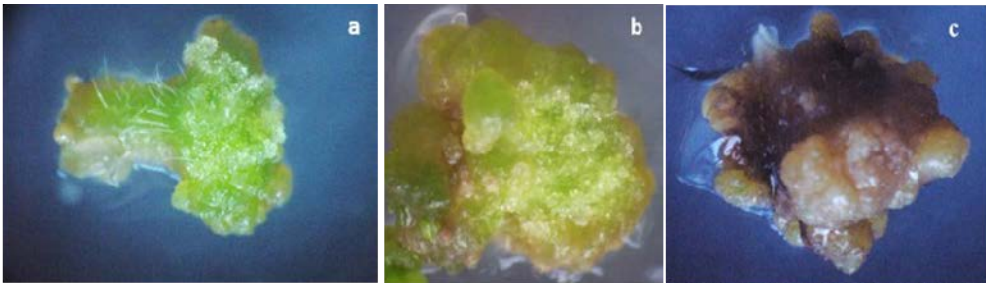

Figure 1. Internodal segments regenerating callus in different conditions: a) an example of polar regeneration of callus on internodal explant wihout drought stress; (b) callus extending on the entire inoculum; (c) callus necrosis under drought stress simulated by 5% PEG.

The callus regenerated on MS-T medium in both transgenic and control potato lines. Callus regeneration began at the apical pole of internodal fragment (Fig. 1 a) and expanded throughout the inoculum (Fig. 1 b). A part of the regenerated calli remained viable after treatment with PEG, but others lost their viability through necrosis (Fig. 1 c) during the first three weeks of testing on RMB5 media with 5% PEG.

Differences between the percentages of viability (%) of calluses derived from transgenic, non-transgenic and control (Désirée and Baltica) potato lines is depicted in Fig. 2. All transgenic genotypes have a higher viability when compared to the control cultivars Désirée and Baltica, but there are also differences between different transgenic genotypes, some performing better than others (Fig. 2).

It is possible that infection with *Agrobacterium* was stimulating organogenesis acting as a stress signal and did also influence plant response to *in vitro* regeneration under stress conditions also in next generations of plants, which means that an epigenetic mechanism should be involved. However, this hypothesis has to be further investigated. From these calluses, plants have been regenerated, under drought stress, in different percentages compared to controls. Based on the rate of regeneration, lines tested for resistance to drought stress were divided into two groups: resistant and susceptible to stress (Fig. 3).

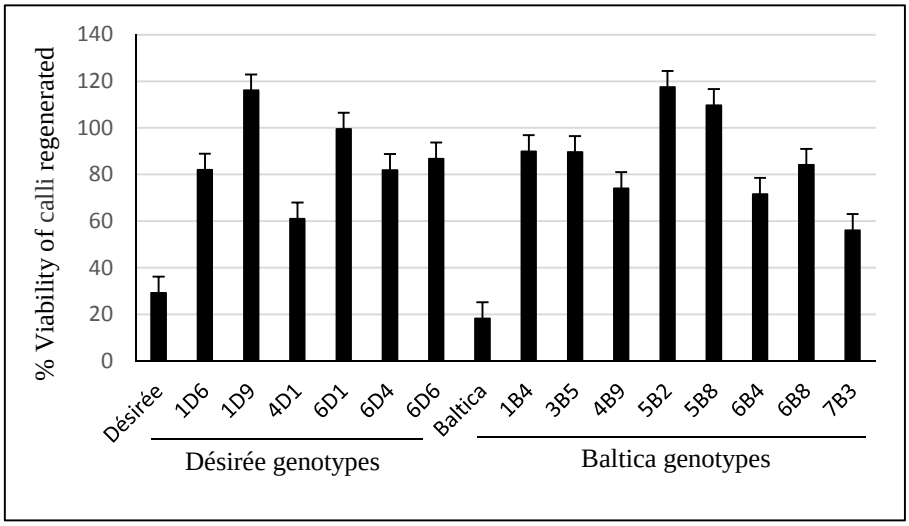

Figure 2. Percentage of viable calluses derived from potato lines transformed with *Agrobacterium* i.e. T1 generation maintained in *in vitro* culture, compared with the controls. Cultivars Désirée and Baltica were used as control. Values are means of percentage of viability of calli regenerated on MS-Tculture media with 5% PEG, n= 5; bars = SE.

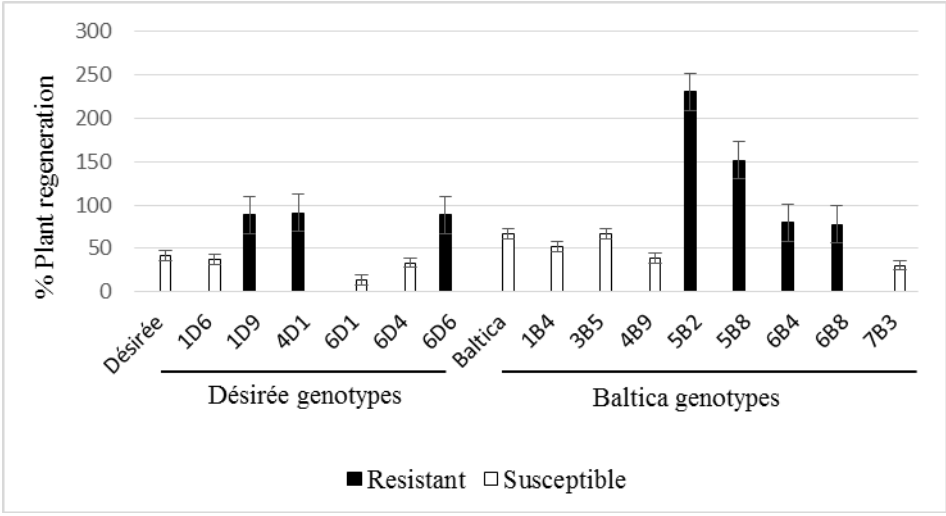

Figure 3. The percentage of potato plant regeneration from calli on MS-T media with 5% PEG, depending on genotype and compared with control cultivar.
The values are means of five replicates; bars = SE.

Thus, lines with a regeneration rate higher than of control lines were considered resistant whereas lines with a regeneration percentage less than or equal to the controls were included in the susceptible group. Following the statistical analysis it was found, that there is a significant difference between these two groups (susceptible and resistant). After PEG induced drought stress, some regenerated shoots suffered necrosis (Fig. 4 a), formed kind of runners (Fig. 4 b) or even vitrified (Fig. 4 c). But some of the plants with integrated PVY resistance gene showed also resistance to drought stress (Fig. 5).

Figure 4. Potato plants regenerated under drought stress conditions from susceptible lines (a - 6D1, b - 1D6, c – 7B3 – exhibits vitrifying leaves).

In the second stage of stress selection, potato lines, which regenerated viable plants from callus under drought stress, were tested on three different concentration 5%, 10%, 15% of PEG 6000 added to the culture media.

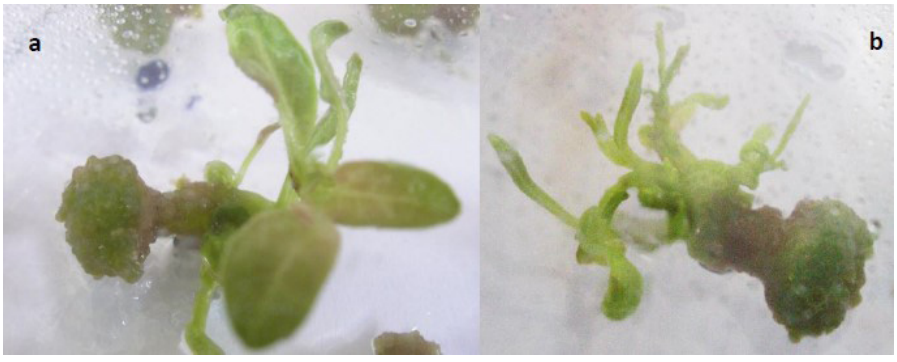

Figure 5. Regenerating plants from drought stress resistant lines (a – line 5B2, b – line 5B8).

Upon selection on 5% PEG, four putative drought resistant lines were selected all belonging to Baltica cultivar (4B9, 5B2, 5B8, 6B4). These lines had higher viability as compared with the control. Also, using T test, it was observed that it is a significant difference between plant viability of the lines 5B2, 5B8, 6B4 and the control wt Baltica cultivated on RMB5 +5% PEG ($p = 0.004 < 0.01$, $p = 0.002 < 0.01$, $p = 0.001 < 0.01$) (Fig. 6).

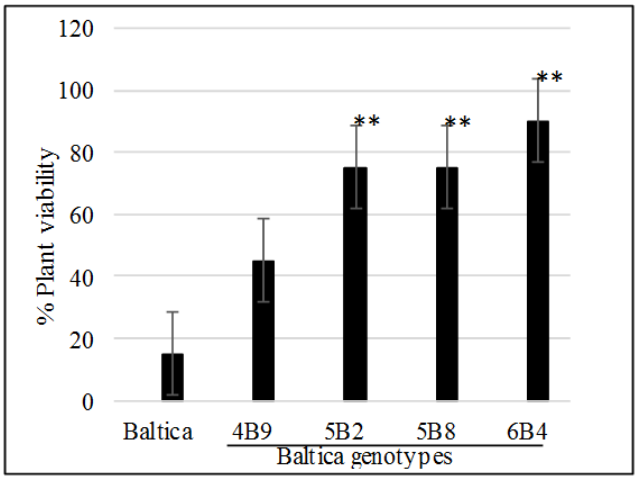

Figure 6. Percentage of plant viability of transgenic and putatively-transgenic genotypes and of control wild-type potato line (Baltica) under drought stress (RMB5 + 5% PEG). There are significant differences between the percentages of plant viability of transgenic and putatively-transgenic genotypes and the control Baltica (** $p < 0.01$). Values are means of percentage of plant viability, n=5, bars=SE

After selection of the four potato lines on RMB5 media supplemented with 10% PEG, the control (Baltica) completely lost viability and line 4B9 had a viability much lower than the other three genotypes (5B2, 5B8, 6B4) that had a percentage of viability over 50% (Fig. 7).

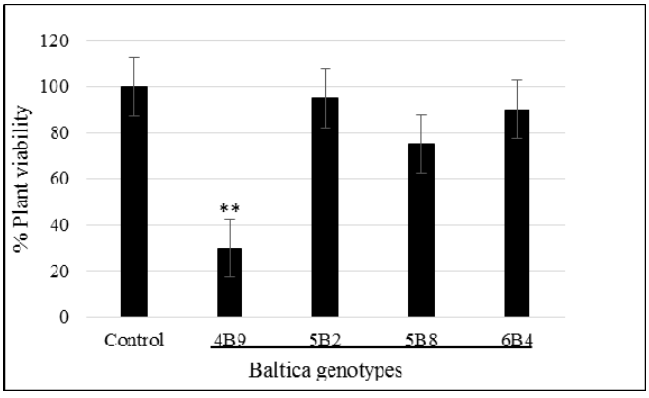

Figure 7. The percentage of plant viability of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 10% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). **Significant difference: $p < 0.01$. Values are means of percentage of plant viability, n=5, bars=SE

Moreover, the statistical analysis showed that the difference between the plant viability of line 4B9 on RMB5 with 10% PEG and the same line on control conditions (RMB5 without PEG), is significant ($p=0.002< 0.01$). At the same time, the difference between the plant viability of lines 5B2, 5B8 and 6B4 under stress conditions (RMB5 + 10% PEG) and control conditions (RMB5 without PEG), is not significant. Thus it was observed that the line 4B9 became susceptible to drought stress (Fig. 8). This aspect was also confirmed during the stress selection on media with 15% PEG, when this line lost its viability. At the same time, during the stress selection on media with 15% PEG, the lines 5B2, 5B8 and 6B4 had percentage of plant viability close to 100% (Fig. 9) and formed roots (Fig. 10) with the average length between 0.5 and 1.5 cm.

The statistical analyses showed that there is no significant difference between the plant viability of these three lines cultivated in both drought stress conditions (RMB5 +15% PEG) and control conditions (RMB5 without PEG).

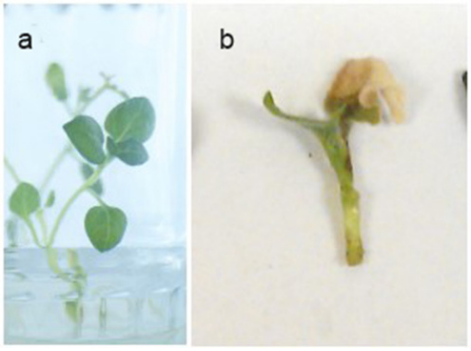

Figure 8. Differences between resistant plant (A - line 5B2) and susceptible plant (B - line 4B9) during the stress selection on RMB5 medium with 10% PEG.

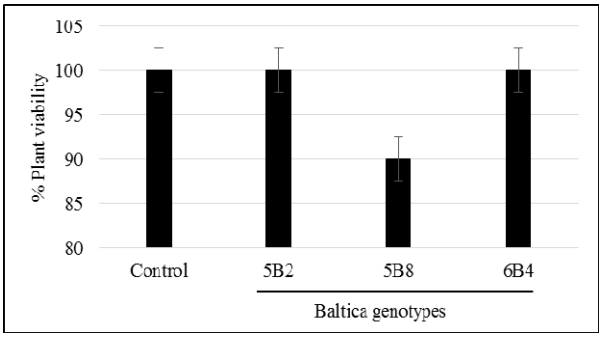

Figure 9. The percentage of plant viability of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 15% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). Values are means of percentage of plant viability, n=5, bars=SE.

After the test with 15% PEG plants have small size (Fig. 11 a, 11 d) but formed roots (Fig. 11 b). These results suggest that *in vitro* stress selection is sufficient to discriminate between susceptible and drought resistant plants in terms of viability and regeneration rate. Our findings are in accordance with previous studies which showed that *in vitro* stress selection is a useful technique to select drought resistant genotypes derived from different plant species such as sunflower (Punia and Jain, 2002), *Rubus* (Orlikowska *et al*., 2009) and potato (Gopal and Iwama, 2007). Previous PCR analysis showed that the five genotypes: 4D1, 6D4, 6D6, 5B2, and 5B8 were positive for the hairpin construct and hence putatively resistant to PVY.

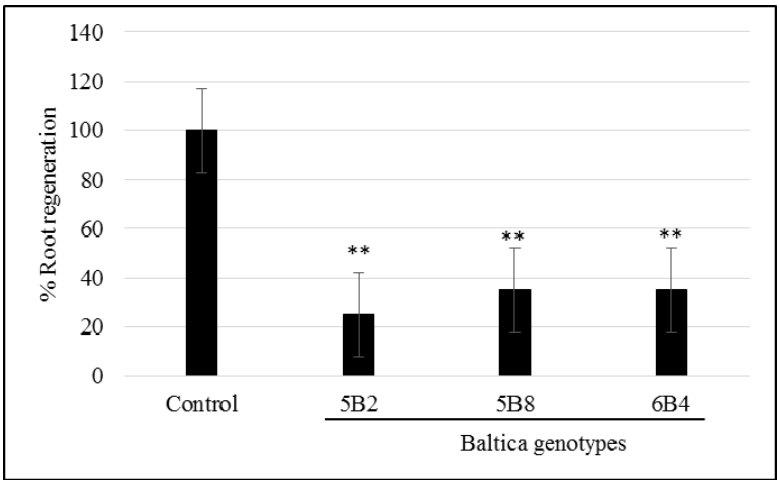

Figure 10. The percentage of root regeneration of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 15% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). There are significant differences between the lines tested on drought conditions and control ($p < 0.01$).
Values are means of percentage of root regeneration, n=5, bars=SE

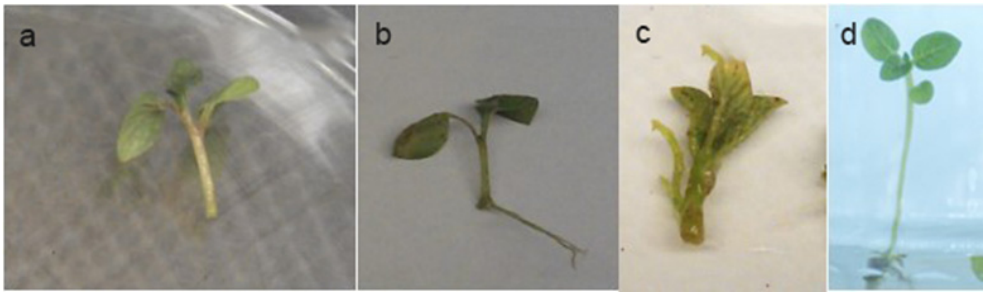

Figure 11. Viable potato plants selected on RMB5 medium with 15% PEG
(a, d – line 6B4; b – line 5B2; c – line 5B8).

In the current experiments, upon applying *in vitro* drought stress selection, two transgenic potato lines 5B2 and 5B8 proved to be also resistant to drought stress determined by PEG in all the three concentrations analyzed at plantlet level (5%, 10% and 15%). In other words, these two genotypes have been induced to carry a double resistance, to biotic (PVY) and abiotic (drought) stress (Table 1).

Table 1.

The response of genotypes undergoing transformation with *Agrobacterium* to drought stress, after all rounds of *in vitro* selections.

Genotype	Transgenic (T) / Not Transgenic (NT)	Resistant (R) / Susceptible (S) to drought stress
Désirée	**Control**	**-**
1D6	**ND***	**S**
1D9	**ND**	**S**
4D1	**T**	**S**
6D1	**ND**	**S**
6D4	**T**	**S**
6D6	**T**	**S**
Baltica	**Control**	**-**
1B4	**ND**	**S**
3B5	**ND**	**S**
4B9	**ND**	**S**
5B2	**T**	**R**
5B8	**T**	**R**
6B4	**ND**	**R**
6B8	**ND**	**S**
7B3	**NT**	**S**

*ND = Not Determined

Conclusions

The results obtained reveal that it is possible to combine transgenesis and *in vitro* stress selection in order to induce more resistant traits in potato, in our case PVY resistance mediated by RNAi and drought resistance.

In addition, it seems that one stress factor already existing in a plant can induce a general mechanism, which prepares the faster response to an additional stress factor.

This strategy might be applied to obtain other potato cultivars or other plant species with resistance to different stress factors.

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