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EFFECT OF COMBINED GROWTH REGULATORS ON *In vitro* MULTIPLICATION OF RECALCITRANT POTATO VARIETIES

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ABSTRACT

The potato varieties studied in this paper were created at the National Institute of Research and Development for Potato and Sugar Beet Braşov. They have a high biological production capacity and are attractive and very appreciated by farmers, potato producers, consumers and processors, but manifest recalcitrance to the *in vitro* culture techniques. Thus we tried to find an optimal culture medium variant for these varieties, in order to obtain healty potato seed material in a short time. The study compared the effects of five different culture media using MS medium containing various concentrations of gibberelic acid (GA_3) in combination with α-naphthalene acetic acid (NAA) in micropropagation of two recalcitrant potato varieties, Marvis and Brasovia. The longest shoots, maximum number of nodes and leaves, the longest roots and the best rooting efficiency were recorded on MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA, for both varieties. The use of a larger quantity of GA_3 negatively influenced the potato shoots and roots growth. The culture media using MS medium containing 4 mgL^{-1} GA_3 and 1 mgL^{-1} NAA had an inhibitory effect on all the studied plantlet characteristics. The results showed that a certain combination of growth regulators (NAA and GA_3) improved the micropropagation capacity of the two recalcitrant potato cultivars studied and resulted in the maximum improvement in the parameters.

Keywords: plant tissue cultures, growth regulators, *in vitro* recalcitrance, potato, variety.

INTRODUCTION

Potato is one of the most important crops in the world and therefore the subject of constant interest and numerous studies including those classified as plant biotechnology (Vinterhalter et. al., 2008). The cultivated potato (*Solanum tuberosum*) ranking fourth in world production after wheat, rice and maize. It is thus the most important dicotyledonous crop species, and the most important tuber crop (Jones, 1994). The development of *in vitro* single-node multiplication systems was a breaking point in the comercial production of high quality potato seed. The first report on the establishment of potato *in vitro* cultures by Stewart and Caplin (1951) was made now more than half a century ago (Vinterhalter et al., 2008). However, there is still plenty of scientific research conducted around the globe studying various aspects of regeneration, physiological responses and production of plantlets. The production of plantlets via nodal cuttings has opened a new area in research on nutrient requirements and nutrient uptake of plantlets during the multiplication stage as well as effects of light and temperature on culturing (Pruski, 2008).

The success of a plant biotechnology project can largely depend upon the ability to regenerate whole plants from *in vitro* cultures (Benson, 2000). *In vitro* recalcitrance is the inability of plant cells, tissues and organs to respond to tissue culture manipulations. With respect to plant regeneration, recalcitrance can be a major limiting factor for the biotechnological exploitation of economically important plant species and it can also impair the wider application of *in vitro* conservation techniques. The three main factors that influence tissue culture responses are: ”whole plant” physiology of the donor; *in vitro* manipulations; *in vitro* plant stress physiology. Integrating our knowledge of whole plant physiology with an understanding of tissue

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culture responses (including the optimization of tissue culture factors) is an essential first step towards overcoming recalcitrance. It is also important to consider that the culture environment *per se* may evoke stress responses which promote recalcitrance problems.

Plants have developed highly complex life cycles and reproductive strategies to competitively secure their place in specific habitats and ensure their survival when challenged by environmental stress brought about by seasonal change. Whole plant life cycle physiology is directly linked to reproduction, vegetative development and morphogenesis. It thus follows that it is one of the most important factors in determining the capacity of explants to respond *in vitro*, and many examples of *in vitro* recalcitrance can be directly attributed to life cycle factors (Benson, 2000).

The successful *in vitro* multiplication of potatoes depends on the presence of a suitable combination of auxins with gibberellic acid (GA_3) in the propagation medium (Kumlay, 2014; Badoni and Chauhan, 2009; Uddin, 2006; Badoni and Chauhan, 2010; Hoque, 2010). In plants, GA_3 is involved physiologically in cell elongation (Levitt, 1974). Therefore, this treatment resulted in increased shoot length (Rabbani et al., 2001). Previous studies show that combined effect of auxins with GA_3 had a positive impact on the shoot and root development of potato plantlets grown *in vitro* (Roest and Bokelmann,1976; Zhang et al., 2005; Badoni and Chauhan, 2009; Danci et al., 2011).

MATERIAL AND METHODS

Several new potato varieties, creations of the Laboratory for Genetic Breeding and Plant Selection, (NIRDPSB Braşov) were introduced in the germplasm collection, at the Research Laboratory for Plant Tissue Cultures. Of these, some varieties behaved very well under *in vitro* conditions (Castrum, Sarmis, Cosiana, Sevastia, Azaria, Darilena, Ervant), and others were recalcitrant (Marvis, Brasovia). In an attempt to find solutions to solve the recalcitrance manifested *in vitro* by certain potato varieties, research has been initiated on the composition of the culture medium, with the purpose of identifying a variant that allows the successful cultivation of these varieties *in vitro* conditions and obtaining vigorous potato plantlets.

Single node cuttings from Marvis and Brasovia, mid-early maturing potato varieties were used in this study. Marvis variety was created at the National Institute of Research and Development for Potato and Sugar Beet Braşov in 2014. The tuber has oval shape, with shallow eyes, yellow skin and light yellow flesh. Regarding the culinary quality, Marvis variety belongs to quality class B, suitable for all types of food dishes. Brasovia variety was created at the same Institute in 2013. The tuber has round-oval shape, shallow eyes, yellow skin and white-yellowish flesh. Regarding the culinary quality, Brasovia variety belongs to quality class A/B, recommended for boiling and salad. It has a very high biological production capacity, obtaining yields over 60 t/ha. The potato variety Brasovia is medium resistant to PVY and PLRV, resistant to wart disease and medium sensitive to late blight on leafes and tubers. It has high production potential, ecological plasticity, production stability, appropriate commercial appearance, being attractive and appreciated by farmers, consumers and processors as well.

Before micropropagation the plantlets belonging to Marvis and Brasovia potato varieties were checked for the PVX, PVS, PVY and PVM viruses. After ELISA testing only healthy plants were used for the study.

Each single node cuttings were aseptically cultured on five growth medium variants. Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) complemented with 2% (w/v) sucrose and 0.9% (w/v) agar was used as control. Different amounts (1 mgL^{-1}, 2 mgL^{-1}, 3 mgL^{-1} and 4 mgL^{-1}) of gibberellic acid (GA_3) and 1 mgL^{-1} of naphthalene acetic acid (NAA) were added to the other four growth medium variants (Table 1).

For a more effective control of microbial contamination, a broad-spectrum product Plant Preservation Mixture (Plant Cell Technology)

that inhibits the growth of pathogens in plant tissue cultures was added to the medium (2 ml/l). The pH of the solution was adjusted at 5.7 with a pH meter. The culture medium was distributed in test tubes, 5 ml each, covered with aluminum foil caps. The sterilization was carried out in the autoclave at temperature of 121°C for 20 minutes.

After inoculation, the cultures were incubated in a growth chamber: temperature 20±2°C, 16:8 photoperiod. During the *in vitro* cultivation several growth parameters were followed: shoot and root length, leaves and nodes number, rooting efficiency and leaf size. The rooting efficiency was scored as 1, 2 and 3 for light, medium and vigorous root growth and the leaf size was scored as 1, 2 and 3 for small, medium and large sized leaves (Rishi et al., 2012).

Table 1. The growth medium variants used for micropropagation of the recalcitrant potato varieties

Variants	Composition	Sucrose (g/l)
V_1	MS (Control)	20
V_2	MS + 1 mgL^{-1} GA_3 + 1 mgL^{-1} NAA	30
V_3	MS + 2 mgL^{-1} GA_3 + 1 mgL^{-1} NAA	30
V_4	MS + 3 mgL^{-1} GA_3 + 1 mgL^{-1} NAA	30
V_5	MS + 4 mgL^{-1} GA_3 + 1 mgL^{-1} NAA	30

For the initiation of the cultures, both aged microplants (about 4 weeks old) and young microplants (about 2-3 weeks old) were used as a source of explants. It was observed that the plantlets regenerated from young explants had a more vigorous growth, the leaves had a larger leaf surface and an intense green color, and the root system was better developed (more and longer roots) compared to the plantlets regenerated from aged explants (Figure 1). The measurements made in this study regarding: shoot and root length, leaves and nodes number, rooting efficiency and leaf size, and the results were achieved on the plantlets obtained from young explants.

Figure 1. Microplants regenerated from young explants (left) and aged explants (right), 1 month after inoculation of explants (Marvis variety)

RESULTS AND DISCUSSION

Adding NAA and GA_3 in culture medium influenced the growth of microplants, number of nodes and leaves, roots length and rooting efficiency. Culture medium variant V_3 (MS + 2 mgL^{-1} GA_3 + 1 mgL^{-1} NAA; 30 g/l sucrose) increased the shoot length, nodes and leaves number, roots length and rooting efficiency compared to control medium MS (Table 2). The use of these hormones (NAA and GA_3) made it possible to obtain the maximum number of nodes (10.73) and leaves (12.03), the longest shoots (8.50 cm) and roots (7.30 cm) and the most vigorous root system (2.17). These results are in agreement with other researchers studies (Webb et al., 1983; Badoni and Chauhan, 2009; Badoni and Chauhan, 2010; Miller et al., 1985; Farhatullah and Sayeed, 2007; Zaman et al., 2001; Dhital et al., 2010).

However, the use of a larger quantity of NAA and GA_3 as in the culture medium variant V_5 (MS + 4 mgL^{-1} GA_3 + 1 mgL^{-1} NAA; 30 g/l sucrose) caused the decrease of the values for parameter as shoot length, nodes and leaves number, roots length and rooting efficiency compared to control medium MS (Table 2). The minimum shoot length (4.95 cm), the lowest number of nodes (7.50) and leaves (8.77), the shortest roots (2.44 cm) and the lightest rooting efficiency (1.17) were noted on culture medium variant with the highest amount of GA_3 (4 mgL^{-1}).

Table 2. Effects of MS medium containing 1 mg L^{-1} NAA and different amounts of GA_3 on shoot and root length, leaves and nodes number, rooting efficiency and leaf size of potato recalcitrant varieties Marvis and Brasovia

Culture medium	Shoot length (cm)	Diff.	Sign.	Number of nodes	Diff.	Sign.
V_1 (Ct)	6.87	-	-	9.17	-	-
V_2	7.40	0.53	ns	10.17	1.00	ns
V_3	8.50	1.63	**	10.73	1.57	*
V_4	7.25	0.38	ns	10.40	1.23	ns
V_5	4.95	-1.92	oo	7.50	-1.67	o
	LSD 5% = 1.10 cm; 1% = 1.51 cm; 0.1% = 2.08 cm			LSD 5% = 1.38 nodes; 1% = 1.90 nodes; 0.1% = 2.62 nodes		

Culture medium	Number of leaves	Diff.	Sign.	Leaf size*	Diff.	Sign.
V_1 (Ct)	10.40	-	-	1.87	-	-
V_2	11.63	1.23	ns	1.93	0.07	ns
V_3	12.03	1.63	*	2.07	0.20	ns
V_4	11.67	1.27	ns	2.23	0.37	ns
V_5	8.77	-1.63	o	1.70	-0.17	ns
	LSD 5% = 1.37 leaves; 1% = 1.89 leaves; 0.1% = 2.60 leaves			LSD 5% = 0.40; 1% = 0.55; 0.1% = 0.76		

Culture medium	Root length (cm)	Diff.	Sign.	Rooting efficiency#	Diff.	Sign.
V_1 (Ct)	4.89	-	-	1.77	-	-
V_2	5.25	0.35	ns	1.87	0.10	ns
V_3	7.30	2.41	**	2.17	0.40	***
V_4	5.95	1.05	ns	1.80	0.03	ns
V_5	2.44	-2.45	oo	1.17	-0.60	ooo
	LSD 5% = 1.45 cm; 1% = 2.00 cm; 0.1% = 2.75 cm			LSD 5% = 0.16; 1% = 0.22; 0.1% = 0.31		

* Leaf size: 1 - small, 2 - medium and 3 - large;
# Rooting efficiency: 1 - light, 2 - medium and 3 - vigorous.

Regarding the shoot length, the variety Marvis was noted with significant positive differences (2.10 cm) on the culture medium to which 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA was added. The minimum shoot length was recorded on variety Brasovia (4.60 cm) using MS medium containing 4 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (Table 3).

Table 3. In vitro growth of two recalcitrant potato varieties microplants on MS medium containing NAA and GA_3

Variety	Shoot length (cm)							
	Marvis (a1)			Brasovia (a2)			a2-a1	Sign.
Variants	Average	Diff.	Sign.	Average	Diff.	Sign.		
V_1 (Ct)	6.67	-	-	7.07	-	-	0.39	ns
V_2	7.51	0.83	ns	7.30	0.23	ns	-0.21	ns
V_3	8.77	2.10	*	8.23	1.17	ns	-0.54	ns
V_4	7.33	0.66	ns	7.17	0.10	ns	-0.17	ns
V_5	5.31	-1.37	ns	4.60	-2.47	oo	-0.71	ns

LSD 5% = 1.55 cm; 1% = 2.14 cm; 0.1% = 2.94 cm LSD 5% = 1.42 cm; 1% = 2.01 cm; 0.1% = 2.96 cm

Plant growth regulators have influenced the number of nodes, so that the maximum number of nodes was obtained on variety Marvis (11.40) on MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (Figure 2). The minimum number of nodes on each variety was obtained on MS medium containing 4 mgL^{-1} GA_3 and 1 mgL^{-1} NAA.

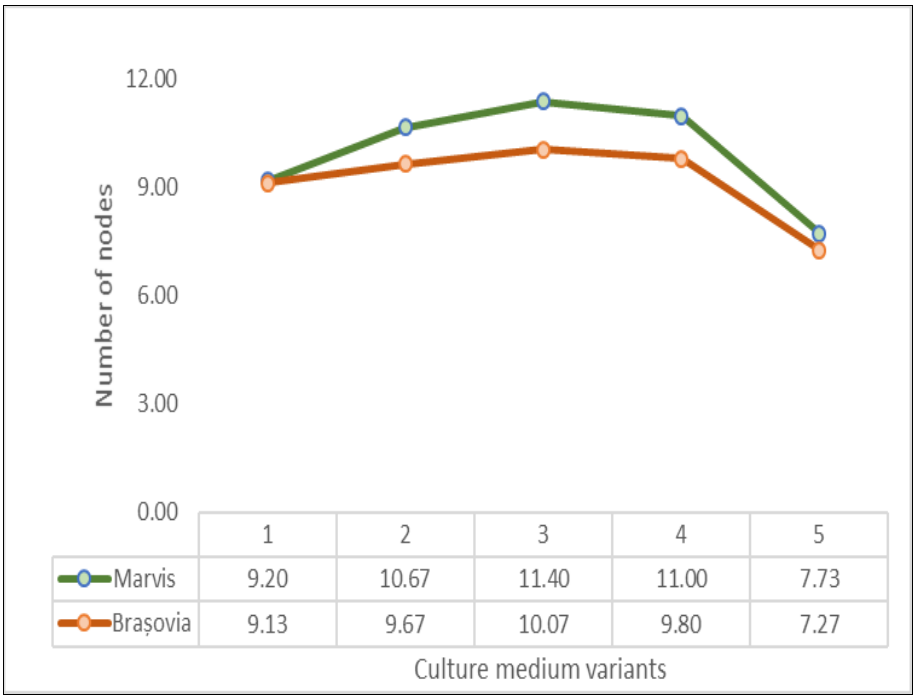

Figure 2. Effect of growth regulators (NAA and GA_3) on number of nodes during *in vitro* cultivation (varieties Marvis and Brasovia)

The number of leaves showed variation after *in vitro* cultivation of recalcitrant potato varieties on different culture medium variants. MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (V_3) resulted in increased number of leaves (12.73) for variety Marvis (Figure 3). The lowest number of leaves for both varieties (9.00 leaves for variety Marvis and 8.53 leaves for variety Brasovia, respectively) was obtained on the V_5 culture medium variant (MS + 4 mgL^{-1} GA_3 + 1 mgL^{-1} NAA; 30 g/l sucrose). The results are in agreement with Kumlay (2014).

Regarding the leaf size results on all five culture medium variants were statistically non-significant for both potato varieties.

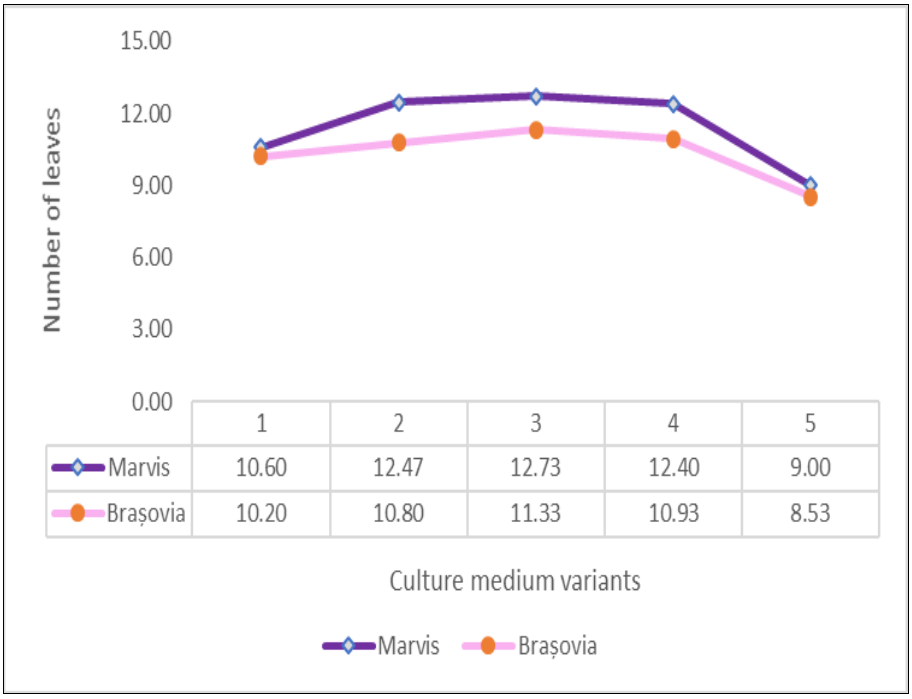

Figure 3. Effect of growth regulators combination (NAA and GA_3) on number of leaves during *in vitro* cultivation of recalcitrant potato varieties Marvis and Brasovia

The plant growth regulators combinations influenced the root length. The longest roots were noted at variety Marvis (7.83 cm) on MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (V_3), followed by variety Brasovia (6.77 cm) on the same culture medium variant (Table 4).

The minimum rooth length on each cultivar (2.85 cm for variety Marvis and 2.03 cm for variety Brasovia, respectively) was noted on MS medium containing 4 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (V_5). V_2 and V_4 variants showed non-significant results.

Table 4. Effect of MS medium containing 1 mg L^{-1} NAA and different amounts of GA_3 on root length of recalcitrant potato varieties Marvis and Brasovia

Variety	Rooth length (cm)							
	Marvis (a1)			Brasovia (a2)			a2-a1	Sign.
Variants	Average	Diff.	Sign.	Average	Diff.	Sign.		
V_1 (Ct)	5.25	-	-	4.54	-	-	-0.71	ns
V_2	5.26	0.01	ns	5.23	0.69	ns	-0.03	ns
V_3	7.83	2.59	*	6.77	2.23	*	-1.07	ns
V_4	6.49	1.25	ns	5.40	0.86	ns	-1.09	ns
V_5	2.85	-2.39	o	2.03	-2.51	o	-0.82	ns
LSD 5% = 2.05 cm; 1% = 2.83 cm; 0.1% = 3.89 cm				LSD 5% = 1.99 cm; 1% = 2.92 cm; 0.1% = 4.85 cm				

A good rooting system increasing the survival rate of plants. The best rooting efficiency was observed in variety Brasovia (2.27), followed by variety Marvis (2.07) on MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA (Table 5). The poorest rooting efficiency among both varieties (1.07 for variety Marvis and 1.27 for variety Brasovia, respectively) was observed on MS medium containing 4 mgL^{-1} GA_3 and 1 mgL^{-1} NAA.

Table 5. Effect of MS medium containing 1 mg L^{-1} NAA and different amounts of GA_3 on rooting efficiency during *in vitro* cultivation (varieties Marvis and Brasovia)

Variety	Rooting efficiency							
	Marvis (a1)			Brasovia (a2)			a2-a1	Sign.
Variants	Average	Diff.	Sign.	Average	Diff.	Sign.		
V_1 (Ct)	1.73	-		1.80	-		0.07	ns
V_2	1.87	0.13	ns	1.87	0.07	ns	0.00	ns
V_3	2.07	0.33	**	2.27	0.47	***	0.20	ns
V_4	1.87	0.13	ns	1.73	-0.07	ns	-0.13	ns
V_5	1.07	-0.67	ooo	1.27	-0.53	ooo	0.20	ns
	LSD 5% = 0.23; 1% = 0.32; 0.1% = 0.44			LSD 5% = 0.30; 1% = 0.54; 0.1% = 1.28				

CONCLUSIONS

Plant reproduction, development and stress all play a role in the natural life cycles of whole plants and it is imperative that we explore the impact that these factors have on our *in vitro* systems. In the future, it will be essential to take an integrated, interdisciplinary approach to develop effective and practical strategies to solve *in vitro* plant recalcitrance problems (Benson, 2000). Micropropagation of potato has been proven to be a very efficient technique to stimulate the high quality production of healthy plants. So it is very important to identify workable solutions that allow efficient *in vitro* multiplication of recalcitrant cultivars.

The results presented here show that the use of gibereline in combination with an auxin is a good way to increase the number of microplants for *in vitro* propagation of potato. These plant growth regulators are of great importance in shoot and root development of *in vitro* cultivated potato. Another observation made in this study was that the plantlets regenerated from young explants had a more vigorous growth and the root system was better developed compared to the plantlets regenerated from aged explants.

In this study, the best results regarding shoot length, number of nodes and leaves, roots length and rooting efficiency were obtained on V_3 culture medium variant. It may concluded that among the five different culture medium variants, the MS medium containing 2 mgL^{-1} GA_3 and 1 mgL^{-1} NAA improved the micropropagation capacity of the two recalcitrant potato cultivars studied and resulted in the maximum improvement in the parameters. It should also be noted that the use of a larger quantity of GA_3 negatively influenced the potato shoots and roots growth. The culture medium variant V_5 (MS + 4 mgL^{-1} GA_3 + 1 mgL^{-1} NAA) had an inhibitory effect on all the studied plantlet characteristics.

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