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In Vitro Conservation and Regeneration of Potato (*Solanum tuberosum* L.): Role of Paclobutrazol and Silver Nanoparticles

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Abstract: In vitro conservation and regeneration of potato germplasm is important in breeding programs. The objective of this study was to assess the combined effect of paclobutrazol (PAC) and silver nanoparticles (AgNPs) during reduced-growth conservation and their in vitro ability to regenerate *S. tuberosum* after conservation treatments. For the conservation system, apices were used as explants in Murashige and Skoog culture media with different combinations of PAC (0, 0.5, 1 and 2 mg L^{-1}) and AgNPs (0, 50, 100 and 200 mg L^{-1}). At six months of culture, plant length, number of lateral branches, number of leaves and roots, root length, stomatal density and total chlorophyll content were assessed. For regeneration, explants were internodes cultured in medium with only 2 mg L^{-1} benzyladenine (BA). At 60 d of culture, the response percentage, number of shoots per explant and shoot length were assessed. For in vitro conservation, the combination of 2 mg L^{-1} PAC and 50 mg L^{-1} AgNPs was the best treatment, whereas for in vitro regeneration, the highest number of shoots was in explants that were treated with 1 or 2 mg L^{-1} PAC with 50 mg L^{-1} AgNPs. In conclusion, PAC and AgNPs are alternatives for in vitro *S. tuberosum* conservation, and their evaluation in other species recalcitrant to the effect of ethylene is recommended.

Keywords: bionanotechnology; chlorophyll; gibberellin inhibitor; ethylene; stomata

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1. Introduction

Potato (*Solanum tuberosum* L.) cultivation contributes to global food security [1,2]. However, factors related to climate change have led to a large in situ loss of biodiversity of this plant genetic resource [3]. In situ and ex situ conservation of potato collections are essential in breeding programs. The disadvantages of these collections are environmental impacts and high pesticide and labor costs to maintain a large number of them [4]. Plant biotechnology offers different alternatives for germplasm conservation through plant tissue culture (PTC), which allows in vitro conservation and the regeneration of germplasm. The in vitro conservation is classified into three categories: short-, medium- and long-term. Medium-term in vitro conservation has more advantages than short-term periods, such as control over storage conditions, and compared to long-term periods that require a constant supply of liquid nitrogen for cryopreservation, which is not cost effective for use in commercial laboratories [5,6]. In addition, medium-term in vitro conservation allows for slow-growth storage, reduces labor costs and allows for the availability of the germplasm bank [7].

Slow-growth storage consists of modifying the physical and chemical conditions of the in vitro culture to reduce the metabolism and development of cells, tissues, organs or whole plants [4]. Physical factors include incubation at low temperatures, the use of nutrient-poor culture media, increased osmotic potential of the culture medium and the use

of growth inhibitors such as ABA and gibberellin synthesis inhibitors (GAs) [8]. The use of GAs inhibitors has been reported in different species such as habanero pepper (*Capsicum chinense* Jacq.) [9], tomato (*Solanum lycopersicum* L.) [10] and potato (*Solanum tuberosum* L.) [11]. Paclobutrazol (PAC) is an inexpensive, low-toxicity, and GAs synthesis inhibitor that can be used for medium-term in vitro conservation by slow growth [12]. In addition, PAC reduces the length of internodes, and facilitates the harvest of fruits due to dwarf plants. However, one of the main problems during medium-term in vitro conservation is maintaining ethylene-sensitive recalcitrant cultures due to the accumulation of this gas in closed vials with limited gas exchange.

To reduce the effects of ethylene during in vitro propagation, there are alternatives such as the use of cyclopropane (CP), 1-methylcyclopropene (1-MCP), 2-aminoethoxyvinyl glycine (AVG) and silver nitrate ($AgNO_3$) that reduce the effects of this gas through different mechanisms. $AgNO_3$ is the most commonly used because silver ions (Ag^+) can reduce the ethylene symptoms of explants by blocking the ethylene receptor, which is the first step during ethylene cell signaling [13]. $AgNO_3$ has been used to counteract the effects of ethylene on crops such as habanero pepper (*Capsicum chinense* Jacq.) [14], tobacco (*Nicotiana tabacum* L.) [15] and potato (*Solanum tuberosum*) [13]. To date, bionanotechnology has enabled the development of metal-based nanomaterials (1–100 nm) such as silver nanoparticles (AgNPs) that could be used to reduce the effects of ethylene [13]. The AgNPs have an effect on shoot proliferation, root–shoot ratio and root elongation [16]. The effects of AgNPs during in vitro conservation have not been fully elucidated. Therefore, the objective of this study was to assess the combined effect of PAC and AgNPs during reduced-growth conservation and their in vitro ability to regenerate *S. tuberosum*.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

In vitro shoots (2.5 cm) regenerated from potato (*Solanum tuberosum* L.) were obtained after three subcultures (every 30 d) in multiplication MS [17] medium supplemented with 2 mg L^{-1} benzylaminopurine (BAP, Sigma®, St. Louis, MO, USA). The medium was solidified with 2.2 g L^{-1} Phytagel™ (Sigma®) and the pH was adjusted to 5.7 before sterilization in an autoclave. The cultures were incubated at 24 ± 2 °C with a white LED light irradiance of 40 ± 5 µmol m^{-2} s^{-1} under a photoperiod of 16 h light.

2.2. Effect of AgNPs and PAC on In Vitro Growth

To evaluate the minimal growth of explants, different concentrations of PAC (0, 0.5, 1 and 2 mg L^{-1}) (CULTAR® 25 SC, Syngenta, Basel, CH, Switzerland) and AgNPs (0, 50, 100 and 200 mg L^{-1}) were evaluated in potato shoot apices (2 cm) as explants. The AgNPs consists of spherical silver nanoparticles with an average diameter of 35 ± 15 nm, with an overall concentration of 20% of AgNPs. The apices were cultured in 22 × 220 mm test tubes containing 20 mL of semisolid MS medium with different concentrations of PAC and AgNPs. The pHs of the culture media and sterilization method were the same as those mentioned above. The test tubes, with one apex each, were incubated under the same conditions as mentioned above. Finally, test tubes with the different treatments were hermetically sealed with cling film (Reynolds® Food Packaging, Lake Forest, IL, USA). After six months of incubation, the shoot length, number of lateral branches, number of leaves and roots, root length, stomatal density and total chlorophyll content were assessed.

2.3. Stomatal Density

Stomatal density (SD) was recorded using the protocol proposed by [18]. The nail-polish imprint method was used over the abaxial leaves. The SD was evaluated through third leaf samples from the apex to the base of the explant in all treatments. The epidermal cells per mm^2 were calculated from five random leaf fields. Leaf samples were analyzed under an optical microscope (M5LCD Velab, Co., Pharr, TX, USA).

2.4. Total Chlorophyll

Chlorophyll content was determined according to the method proposed by [19]. For all experiments, 0.2 g of fresh matter was used and macerated using 75% acetone. The samples were stood at −4 °C for 24 h. Afterwards, the mixture was sieved through filter paper, then adjusted to a volume of 12.5 mL with 75% acetone. The mixture was adjusted to 6.25 mL with 75% acetone and filtered with paper. Finally, absorbance was measured in a spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA).

2.5. In Vitro Regeneration

After six months of in vitro conservation, individual 2–3 cm long shoots obtained in all treatments were cultured in multiplication medium for 30 d. The culture medium was prepared according to Adly et al. [13] and consisted of semisolid MS medium supplemented with 2 mg L^{-1} BAP. The medium was solidified using 2.2 g L^{-1} Phytagel™ and the culture conditions were the same as in the multiplication stage.

2.6. Data Analysis and Experimental Design

All of the experiments were distributed in a completely randomized design using 10 explants per treatment and replicated three times. For response (%), the data were transformed with the formula Y = arcsine($\sqrt{x}/100$), where "x" is the percentage value. Data were subjected to an analysis of variance with a comparison of means using Tukey's test ($p < 0.05$). The Statistical Package for the Social Sciences®, v22 for Windows statistical package was used.

3. Results

3.1. Effect of AgNPs and PAC on Physiological and Biochemical Parameters

When evaluating the effect of the different concentrations of PAC and AgNPs on the development variables, significant differences were found between the treatments. In addition, an interaction between PAC and AgNPs was found for all of the development variables evaluated (Table 1). The longest explants were found in the treatments without PAC and with 0, 50 or 100 mg L^{-1} of AgNPs, being 15 ± SE cm in length, whereas the shortest explants were obtained in the treatments in combination with 2 mg L^{-1} PAC and with 50, 100 or 200 mg L^{-1} of AgNPs, being 5 ± SE cm in height. For the variable number of lateral branches, the highest number of branches was obtained in the treatment with PAC and with 0 or 50 mg L^{-1} of AgNPs, having 19 ± SE branches on average per explant, whereas the lowest number of lateral branches was obtained in the treatments in combination with 1 or 2 mg L^{-1} of PAC and with 50 or 100 mg L^{-1} of AgNPs, and 2 mg L^{-1} of PAC supplemented with 200 mg L^{-1} of AgNPs. Regarding the number of leaves per explant, the highest number of leaves was observed in the treatments with PAC alone, with 27 ± SE leaves per explant, whereas the lowest number of leaves per explant was observed in the treatments supplemented with 2 mg L^{-1} of PAC and with 50, 100 or 200 mg L^{-1} of AgNPs, with 11 ± SE leaves per explant. Regarding the number of roots per explant, the highest number of roots was observed in the treatments with PAC without AgNPs, and 50, 100 and 200 mg L^{-1} of AgNPs without PAC, with 10 ± SE, whereas the lowest number of roots was observed in the treatments with 2 mg L^{-1} of PAC and with 50, 100 or 200 mg L^{-1} of AgNPs in combination, with 6 ± SE roots per explant. Regarding the root length, the longest roots were observed in the treatments in combination with 0 or 0.5 mg L^{-1} of PAC and 0, 50 or 100 mg L^{-1} of AgNPs, being 11 ± SE cm in length, whereas the shortest roots were observed in the treatments with 2 mg L^{-1} of PAC and supplemented with 50, 100 or 200 mg L^{-1} of AgNPs, being 5 ± SE cm in length (Figure 1). Regarding the stomatal density, the highest densities were observed in the treatments supplemented with 2 mg L^{-1} of PAC and with 50, 100 or 200 mg L^{-1} of AgNPs, with 289.25 ± SE, 287.25 ± SE and 289.25 ± SE, respectively, whereas the lowest stomatal density was observed in the treatments with 0, 0.5 and 1 mg L^{-1} of PAC and supplemented with 0 mg L^{-1} of AgNPs, with 194.25 ± SE, 195.50 ± SE and 194.50 ± SE, respectively, and with PAC and with 50 mg L^{-1} of AgNPs,

with 191.75 (Figure 2). As for chlorophyll content, the highest contents were observed in the treatments with PAC supplemented with 50 mg L^{-1} of AgNPs, 2 mg L^{-1} of PAC supplemented with 100 or 200 mg L^{-1} of AgNPs, having 6 ± SE mg g^{-1} FW, whereas the lowest chlorophyll contents were observed in the control treatment (0 mg L^{-1} PAC and 0 mg L^{-1} AgNPs) and with only PAC, having 0.13 ± SE mg g^{-1} FW, without PAC and 50 mg L^{-1} of AgNPs.

Table 1. Effect of PAC and AgNPs on development variables during in vitro conservation of potato (*Solanum tuberosum* L.).

PAC (mg L^{-1})	AgNPs (mg L^{-1})	Shoot Length (cm)	No. of Lateral Branches	No. of Leaves Per Explant	No. of Roots Per Explant	Root Length (cm)	Stomatal Density	Total Chl (mg g^{-1} FW)
0	0	15.80 ± 0.19 a	19.95 ± 0.40 a	26.85 ± 0.89 a	10.20 ± 0.28 a	11.85 ± 0.24 a	194.25 ± 7.02 d	0.13 ± 0.00 c
0.5		10.90 ± 0.48 bc	19.90 ± 0.44 a	26.80 ± 1.00 a	10.30 ± 0.29 a	11.35 ± 0.22 a	195.50 ± 7.35 d	0.14 ± 0.00 c
1		9.45 ± 0.40 c	19.65 ± 0.56 a	28.60 ± 0.93 a	10.65 ± 0.27 a	7.00 ± 0.16 b	194.50 ± 4.57 d	0.14 ± 0.00 c
2		7.69 ± 0.49 cd	19.44 ± 0.40 a	27.55 ± 1.05 a	10.11 ± 0.29 a	6.83 ± 0.20 b	195.75 ± 5.35 cd	0.13 ± 0.00 c
0	50	15.66 ± 0.22 a	19.00 ± 0.41 a	26.00 ± 0.90 ab	10.55 ± 0.29 a	11.70 ± 0.24 a	191.75 ± 6.56 d	0.12 ± 0.00 c
0.5		15.23 ± 0.30 ab	9.10 ± 0.42 b	24.26 ± 1.14 ab	9.78 ± 0.32 ab	7.00 ± 0.20 b	235.00 ± 7.35 b	0.58 ± 0.02 a
1		7.22 ± 0.21 cd	6.55 ± 0.37 c	17.70 ± 0.85 d	8.00 ± 0.34 cd	7.15 ± 0.18 b	246.75 ± 4.49 b	0.59 ± 0.03 a
2		5.58 ± 0.19 e	4.83 ± 0.32 c	11.77 ± 0.67 e	6.72 ± 0.26 d	5.50 ± 0.23 c	289.25 ± 1.88 a	0.64 ± 0.02 a
0	100	15.8 ± 0.23 a	9.47 ± 0.38 b	22.21 ± 1.36 bc	10.00 ± 0.35 a	11.26 ± 0.26 a	230.25 ± 8.29 bc	0.54 ± 0.03 ab
0.5		7.14 ± 0.23 d	9.41 ± 0.45 b	17.23 ± 0.87 d	9.64 ± 0.35 ab	11.11 ± 0.26 a	241.50 ± 6.84 b	0.44 ± 0.02 b
1		6.71 ± 0.15 de	6.31 ± 0.38 c	17.47 ± 0.69 d	8.10 ± 0.34 cd	7.31 ± 0.18 b	240.75 ± 6.15 b	0.53 ± 0.03 ab
2		5.40 ± 0.23 e	4.62 ± 0.35 c	11.68 ± 0.64 e	6.75 ± 0.28 d	5.56 ± 0.30 c	287.25 ± 4.06 a	0.61 ± 0.02 a
0	200	14.95 ± 0.29 ab	8.90 ± 0.49 b	18.80 ± 0.77 cd	9.95 ± 0.32 a	6.95 ± 0.19 b	231.00 ± 8.22 bc	0.54 ± 0.02 ab
0.5		8.70 ± 0.38 c	9.45 ± 0.38 b	18.10 ± 0.70 cd	8.30 ± 0.31 bc	6.90 ± 0.19 b	237.50 ± 9.81 b	0.54 ± 0.02 ab
1		6.66 ± 0.25 de	9.42 ± 0.42 b	18.10 ± 0.69 cd	8.00 ± 0.35 cd	6.84 ± 0.19 b	245.75 ± 11.34 b	0.52 ± 0.03 ab
2		5.57 ± 0.17 e	5.47 ± 0.24 c	11.36 ± 0.39 e	6.68 ± 0.27 d	5.15 ± 0.25 c	289.25 ± 4.21 a	0.62 ± 0.01 a
p-value								
p (PAC)		0.000	0.000	0.000	0.000	0.000	0.000	0.000
p (AgNPs)		0.000	0.000	0.000	0.000	0.000	0.000	0.000
p (PAC × AgNPs)		0.000	0.000	0.000	0.000	0.000	0.000	0.000

Results are expressed as mean ± SE (standard error). Different letters indicate statistical significance according to Tukey's test ($p < 0.05$) between columns. PAC: paclobutrazol; AgNPs: silver nanoparticles.

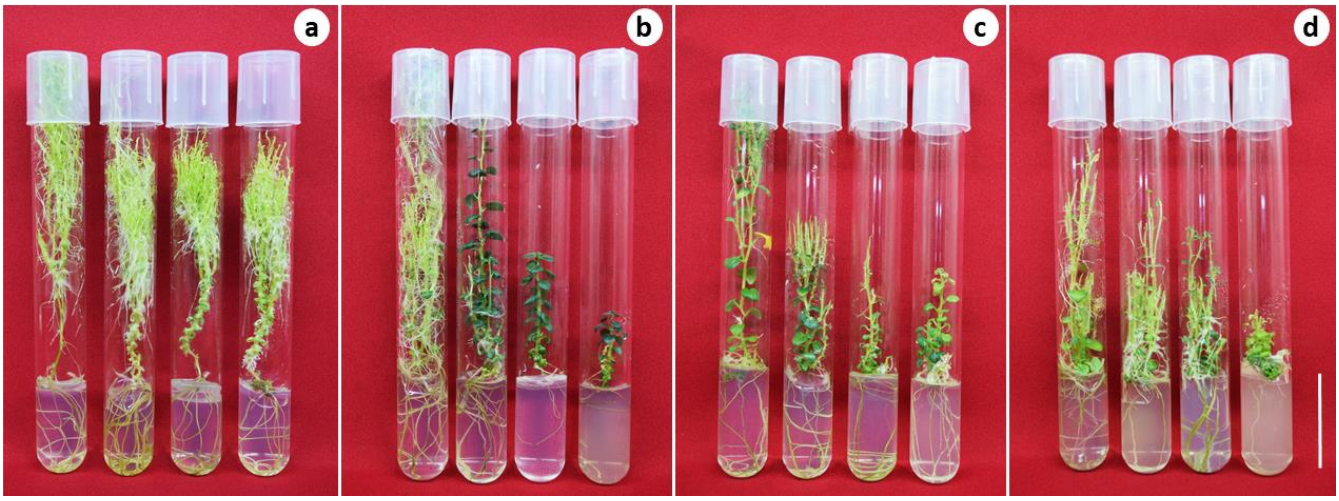

Figure 1. Effect of the concentration of PAC and AgNPs on in vitro conservation of *Solanum tuberosum* L., at six months of culture. Concentrations from left to right in each figure: (**a**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 0 mg L^{-1} of AgNPs; (**b**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 50 mg L^{-1} of AgNPs; (**c**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 100 mg L^{-1} of AgNPs; and (**d**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 200 mg L^{-1} of AgNPs. White bar = 5 cm.

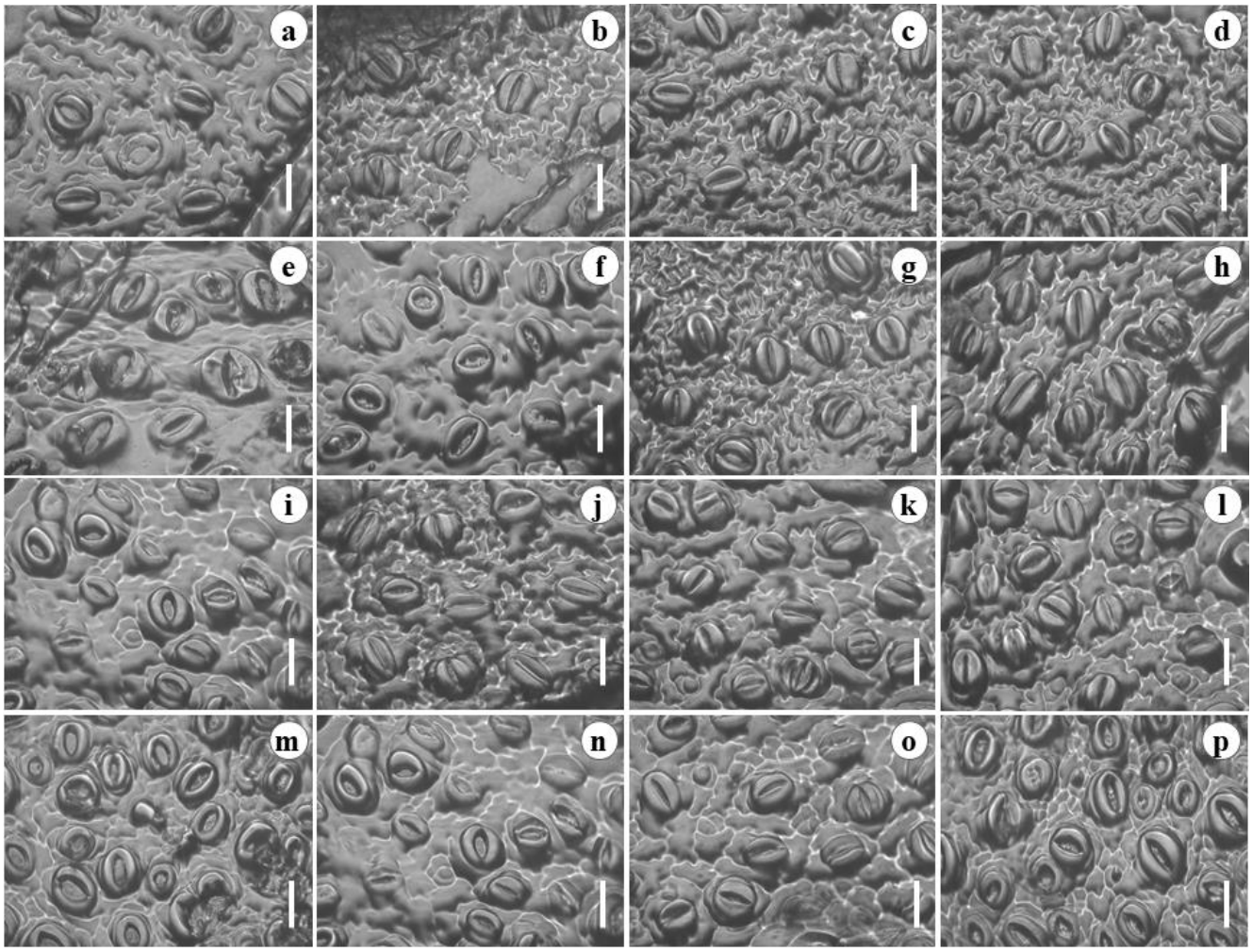

Figure 2. Effect of the concentration of PAC and AgNPs on stomatal density during in vitro conservation of *Solanum tuberosum* L. Concentrations from left to right in each figure: (**a**–**d**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 0 mg L^{-1} of AgNPs; (**e**–**h**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 50 mg L^{-1} of AgNPs; (**i**–**l**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 100 mg L^{-1} of AgNPs; and (**m**–**p**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 200 mg L^{-1} of AgNPs (40× magnification). White bar = 50 µm.

3.2. In Vitro Regeneration after PAC and AgNP Treatments

When evaluating the effect of in vitro shoot multiplication after conservation with PAC and AgNPs, differences were found in the response percentage and number of shoots per explant; however, no differences were observed for the variable shoot length. In addition, an interaction was observed for the variable's response percentage and number of shoots in the explants obtained in the treatments with PAC and AgNPs; however, no interaction was observed for the variable shoot length between the explants obtained in the treatments with PAC and AgNPs (Table 2). The highest response percentage was obtained in the treatments with 1 or 2 mg L^{-1} of PAC supplemented with 50 mg L^{-1} of AgNPs, with 100% response, whereas the lowest response percentage was obtained in the treatments 0 and with PAC without AgNPs, and without PAC supplemented with 50 mg L^{-1} of AgNPs, with 37 to 62% response. For the variable number of shoots per explant, the highest number of shoots was obtained in the treatments with 1 or 2 mg L^{-1} of PAC supplemented with 50 mg L^{-1} of AgNPs, with 4.6 shoots per explant, whereas the lowest number of shoots per explant was obtained in the rest of the treatments. As for the variable shoot length, no significant differences were observed between the length of the shoots obtained after the conservation treatments with PAC and AgNPs (Figure 3).

Table 2. In vitro multiplication of potato (*Solanum tuberosum* L.) after in vitro conservation using PAC and AgNPs.

PAC (mg L^{-1})	AgNPs (mg L^{-1})	Response (%)	No. of Shoots Per Explant	Shoot Length (cm)
0	0	43.75 ±12.50 c	2.30 ± 0.26 b	3.78 ± 0.24 a
0.5		43.75 ± 12.50 c	2.16 ± 0.40 b	3.97 ± 0.30 a
1		37.50 ± 14.43 c	2.00 ± 0.36 b	3.55 ± 0.28 a
2		37.50 ± 14.43 c	2.16 ± 0.24 b	3.64 ± 0.27 a
0	50	62.50 ± 14.43 c	2.07 ± 0.22 b	3.34 ± 0.31 a
0.5		93.75 ± 12.50 ab	2.93 ± 0.23 b	3.08 ± 0.17 a
1		100.00 ± 0.00 a	4.66 ± 0.18 a	3.42 ± 0.10 a
2		100.00 ± 0.00 a	4.66 ± 0.18 a	3.34 ± 0.10 a
0	100	62.50 ± 14.43 bc	2.00 ± 0.44 b	3.95 ± 0.28 a
0.5		87.50 ± 14.43 ab	3.00 ± 0.30 b	3.07 ± 0.16 a
1		87.50 ± 14.43 ab	3.36 ± 0.33 ab	3.56 ± 0.13 a
2		93.75 ± 12.50 ab	3.21 ± 0.31 ab	3.45 ± 0.24 a
0	200	87.50 ± 14.43 ab	2.00 ± 0.57 b	3.00 ± 0.35 a
0.5		68.75 ± 12.50 bc	2.37 ± 0.18 b	2.85 ± 0.21 a
1		87.50 ± 14.43 ab	2.87 ± 0.22 b	3.30 ± 0.18 a
2		81.25 ± 12.50 abc	2.76 ± 0.23 b	2.94 ± 0.16 a
p-value				
p (PAC)		0.000	0.000	0.386
p (AgNPs)		0.005	0.000	0.001
p (PAC × AgNPs)		0.002	0.000	0.465

Results are expressed as mean ± SE (standard error). Different letters indicate statistical significance according to Tukey's test ($p < 0.05$) between columns. PAC: paclobutrazol; AgNPs: silver nanoparticles.

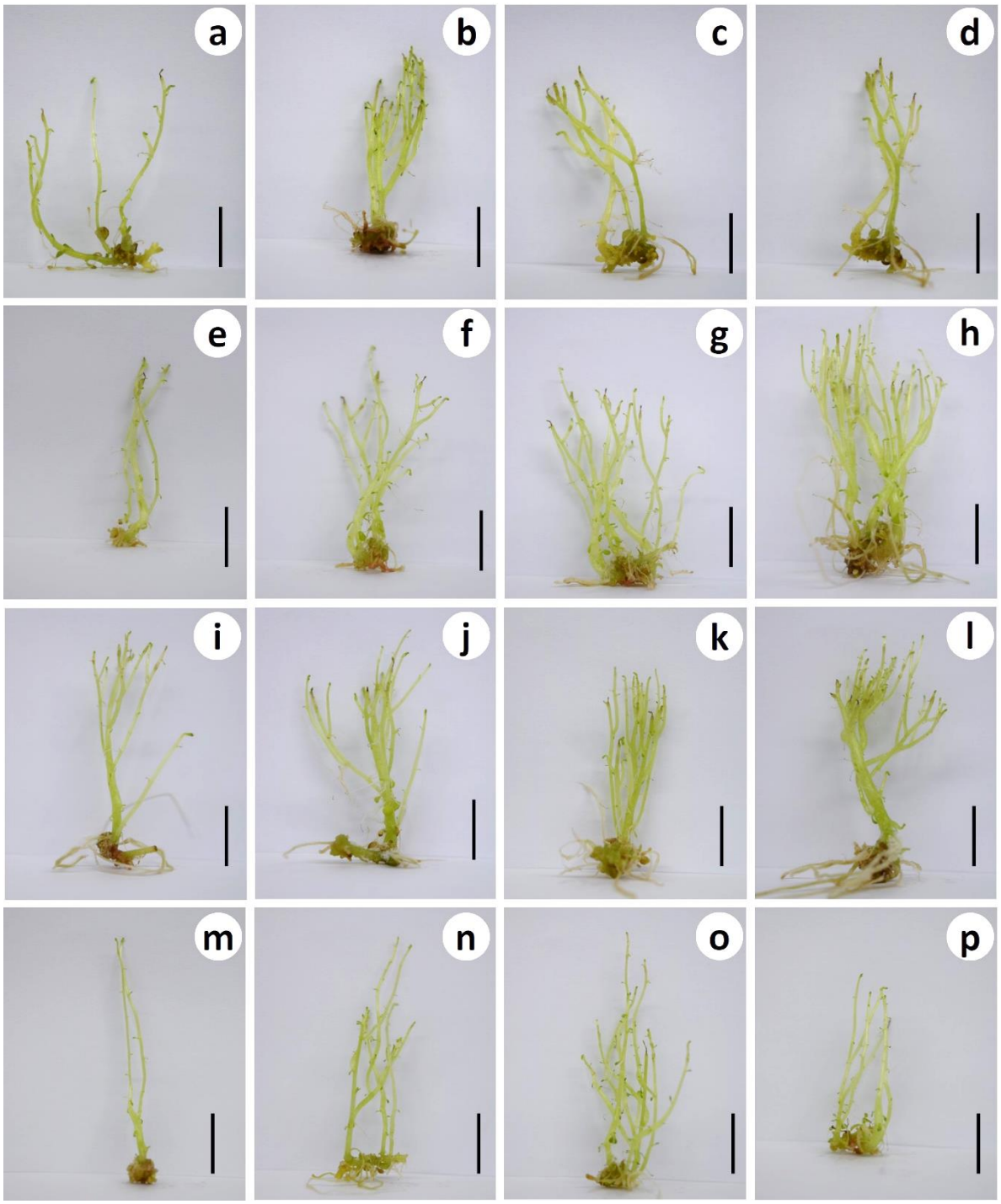

Figure 3. In vitro multiplication of *Solanum tuberosum* L. after PAC and AgNPs treatments at 60 d of culture. Concentrations from left to right in each figure: (**a**–**d**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 0 mg L^{-1} of AgNPs; (**e**–**h**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 50 mg L^{-1} of AgNPs; (**i**–**l**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 100 mg L^{-1} of AgNPs; and (**m**–**p**) 0, 0.5, 1 and 2 mg L^{-1} of PAC + 200 mg L^{-1} of AgNPs. Black bar = 5 cm.

4. Discussion

4.1. Effect of PAC and AgNPs on Physiological and Biochemical Parameters

This study demonstrates the effect of different concentrations of PAC and AgNPs on the variables evaluated during the in vitro conservation of *S. tuberosum*. In vitro conservation using gibberellin inhibitors (GAs) is an alternative to maintain genetic resources in the

medium term. Triazole-type GA inhibitors, such as PAC, possess a nitrogen-containing heterocyclic ring with free electrons that allows the inactivation of monooxygenase enzymes and prevents the transition from ent-caurene to ent-caurenoic acid, thus inhibiting the synthesis of all gibberellins. The use of GA inhibitors in in vitro conservation has been reported in sugarcane (*Saccharum* spp.) [7,20], potato (*S. tuberosum*) [11], native orchid (*Stanhopea tigrina* Bateman ex Lind) [21] and anthurium (*Anthurium andraeanum* L.) [7].

In this study, shoot length decreased as PAC concentrations increased. In addition, PAC failed to inhibit lateral branch growth, leaf number, root number and chlorophyll content. However, when PAC was combined with AgNPs, explants had reduced in lateral growth and increased in leaf number, root number, stomatal density and chlorophyll content. This is probably because the ions (Ag^+) are not directly involved in plant development, but they could have an effect through ethylene sensitivity, whereas PAC generates a cellular compaction and accumulation of photosynthetic pigments due to a packing of chloroplasts in the cell tissue. Regarding root length, the smallest roots were observed in the treatments with 1 and 2 mg L^{-1} of PAC. Spinoso-Castillo et al. [7] found an increased number of shoots in sugarcane during in vitro conservation at a concentration of 1 mg L^{-1} of PAC. Zeid et al. [11] observed an increased number of microtubers per explant in *S. tuberosum* during in vitro conservation at a concentration of 3 mg L^{-1} of PAC.

Silver has been used to counteract the effects of ethylene in habanero pepper (*Capsicum chinense* Jacq.) [14], stevia (*Stevia rebaudiana* B.) [22] and potato (*Solanum tuberosum*) [13,23]. Kaur and Kumar [23] found an increase in shoot regeneration during the in vitro propagation of *S. tuberosum* at a concentration of 0.28 mg L^{-1} $AgNO_3$. Adly et al. [10] found growth stimulation during in vitro propagation of *S. tuberosum* at a concentration of 4 mg L^{-1} of $AgNO_3$. Adly et al. [13] state that this effect can be explained by interference with ethylene perception to decrease the effect of the accumulation of the gas hormone during in vitro culture. In comparison with $AgNO_3$, AgNPs release ions (Ag^+) that can block the ethylene receptor by replacing the copper ion cofactor of the ethylene binding site. According to Elatafi and Fang [24], AgNPs have a larger surface area to volume ratio in comparison to other silver forms such as $AgNO_3$, which may make them more effective, while they also have lower toxicity effects. In addition, the ions (Ag^+) of AgNPs possess the property of inhibiting the formation of 1-aminocyclopropane-1-carboxylic acid and, consequently, the action of ethylene [25].

The interaction of PAC and AgNPs at different concentrations during the in vitro conservation of potato had effects on stomatal density (SD) and chlorophyll content. The SD is a physiological characteristic that measures the number of stomata per mm^2, and is influenced by RH, water stress and light. However, in this study, SD was influenced by cell compaction per mm^2. Variation in stomatal density using PAC has been reported in different plant species such as sugarcane (*Saccharum* spp.), anthurium (*Anthurium andraeanum*) and agave (*Agave potatorum*) [7]. Spinoso-Castillo et al. [7] found an increase in stomatal density during the in vitro conservation of *Saccharum* spp., *A. andraeanum* and *A. potatorum* at a concentration of 3 mg L^{-1} PAC. The cell size could be due to an inhibition of cell elongation caused by PAC, and probably due to decreased ethylene effects in tissues by AgNPs, or due to the combined effect of PAC × AgNPs. Ethylene accumulation in closed culture vessels in in vitro culture stimulates the endoreduplication of epidermal cells [26], thus increasing the density and unorganized cells in the leaves of the in vitro explants. On the other hand, variation in stomatal density using AgNPs has been reported in *Gaillardia pulchella* Foug cv Torch Yellow [27]; Manokari et al. [27] found a decrease in stomatal density during the in vitro conservation of *G. pulchella* at a concentration of 4 mg L^{-1} of AgNPs.

Regarding the variation in chlorophyll content in the PAC and AgNPs treatments, concentrations of 2 mg L^{-1} of PAC supplemented with 50, 100 or 200 mg L^{-1} of AgNPs showed an increase in chlorophyll content. AgNPs could improve photosynthetic efficiency due to a stimulating effect on the photosystems I and II that carries out the light reaction and enhancing electron transfer like a conductor that intensifies the photosynthesis pro-

cesses, while PAC increases the relative chlorophyll content per unit leaf area. Variation in chlorophyll content using PAC has been reported in sugarcane (*Saccharum* spp.), anthurium (*Anthurium andraeanum*) and agave (*Agave potatorum*) [7]. Spinoso-Castillo et al. [7] found an increase in chlorophyll content during the in vitro conservation of *Saccharum* spp., *A. andraeanum* and *A. potatorum* at concentrations of 1 and 2 mg L^{-1} of PAC. Furthermore, this increase in total chlorophyll content is probably due to the relative increase in the number of stomata and epidermal cells in compacted leaves. On the other hand, variation in chlorophyll content using AgNPs has been reported in different plant species such as vanilla (*Vanilla planifolia*) [28], strawberry (*Fragaria* × *ananassa*) [29], tobacco (*Nicotiana tabacum* L.) [15] and grape (*Vitis vinifera* L.) [24]. Spinoso-Castillo et al. [28] found that *V. planifolia* seedlings at doses of 25 to 200 mg L^{-1} of AgNPs showed the highest in vitro chlorophyll values. Tung et al. [29] found that *Fragaria* × *ananassa* seedlings at a dose of 0.20 mg L^{-1} of AgNPs showed the highest in vitro chlorophyll values. Spinoso-Castillo et al. [28] and Castro-González et al. [22] reported an increase in chlorophyll content during the in vitro multiplication of vanilla and stevia shoots treated with AgNPs; this effect was probably due to the increase in N, Mg and Fe concentrations in the explant exposed to AgNPs, since these elements are related to chlorophyll biosynthesis.

4.2. In Vitro Regeneration after PAC and AgNP Treatments

In this study, it was possible to regenerate shoots from *S. tuberosum* seedlings obtained during in vitro conservation using different concentrations of PAC and AgNPs. The most efficient reduced-growth conservation systems are those that do not affect the regeneration ability once they are transferred from multiplication. Spinoso-Castillo et al. [7] regenerated shoots from plantlets after the in vitro conservation of *Saccharum* spp., *A. andraeanum* and *A. potatorum* with different treatments of PAC. In our study, shoot regeneration after conservation treatments with 1 and 2 mg L^{-1} of PAC in combination with 50 mg L^{-1} of AgNPs is probably due to a residual effect of these concentrations contributing to a better regeneration ability. According to Kumar et al. [30], PAC reaches a residual effect of three months in tissues after application. The supplementation of AgNPs during the in vitro conservation of ethylene-sensitive species could also be useful for regeneration due to a probable residuality of ions (Ag^{+}) within the tissues. Castro-González et al. [22] found that AgNPs in stevia (*S. rebaudiana*) shoots were transported from the culture medium and accumulated within plant tissues.

The combined effect of PAC on the inhibition of cell elongation and of AgNPs to counteract ethylene symptoms showed different responses on the variables evaluated. The in vitro conservation of *S. tuberosum* would be more efficient with the addition of PAC acting as a growth inhibitor, this compound thereby increasing the incubation period and meaning that less manipulation is necessary, in addition to costs associated with materials and labor. Spinoso-Castillo et al. [7] state that this conservation system is low-cost due to the low price of PAC. In addition, AgNPs are an efficient alternative due to their ability to inhibit the effects of ethylene to promote vegetative growth in crops sensitive to this gas. Castro-González et al. [22] suggested the use of silver nanosilver at concentrations lower than 100 mg L^{-1} in order to avoid long-term effects and reduced cost during in vitro propagation. This protocol only requires two subcultures per year for *S. tuberosum*. Between each subculture (six months), the explants remain alive and can be used at any time for breeding programs. Furthermore, no visible anatomical changes were observed in regenerated shoots. However, future somaclonal variation could be analyzed, and studies should be conducted on silver nanoparticles, focusing on residual particles in tissues, and genotoxic effects during medium-term in vitro conservation.

5. Conclusions

In conclusion, it was determined that the supplementation of PAC and AgNPs to MS culture medium affected development, chlorophyll content and stomatal density in potato seedlings during in vitro conservation at six months of culture. The combination of 2 mg

L^{-1} of PAC and 50 mg L^{-1} of AgNPs was the best treatment for conservation, whereas the post-conservation treatments of 1 and 2 mg L^{-1} of PAC supplemented with 50 mg L^{-1} of AgNPs were the best combinations for multiplication. In addition, seedlings obtained using this method can be transferred to shoot multiplication medium. Therefore, PAC and AgNPs are a low-cost alternative for in vitro potato conservation, and their evaluation in other ethylene-sensitive species is recommended.

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