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Aeration and Chemical Additives Prevent Hyperhydration and Allow the Production of High-Quality In Vitro Potato Plantlets

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Abstract: The production of healthy propagating material of the potato (*Solanum tuberosum* L.) is based on in vitro micropropagation. In vitro conditions, however, can cause stress leading to reduced quality, growth and development of in vitro plantlets. The effects of aeration and chemical additives on the in vitro growth and development and quality of potato plantlets were investigated. Four different jar closure types were tested, i.e., an intact metal cap (control), two layers of semi-permeable plastic foil, a metal cap with a single hole, or a metal cap with three holes. Under tightly sealed conditions (intact metal cap) the effects of silver nitrate (2.0 mg L^{-1}) and 1-naphtylacetic acid (0.1 mg L^{-1}) alone or in combination with each other, meta-topoline (0.1 mg L^{-1}), ascorbic acid (10.0 mg L^{-1}), salicylic acid (0.1 mg L^{-1}), jasmonic acid (0.1 mg L^{-1}) and glutamic acid (0.3 mg L^{-1}) were studied. Morpho-physiological parameters were measured at the end of the subculture. Leaf development was a good indicator of the presumed ethylene effect. The development and quality of the plantlets were best in cultures sealed with three-holed caps. Of the chemicals applied, only the presence of silver nitrate resulted in high-quality plantlets. The combined application of silver nitrate and 1-naphthaleneacetic acid promoted root growth and development.

Keywords: ethylene; leaf development; plant quality; rooting; silver nitrate; stress; tissue culture

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1. Introduction

Plant tissue cultures (PTCs) need to be isolated from the external environment to maintain them under sterile conditions and avoid desiccation of the plant material. However, the environment in a PTC is basically different from the natural environment of plants [1]. A number of abiotic stress factors, including mainly high humidity, carbon dioxide (CO_2) level, endogenously produced ethylene (ETH) and other organic products, the type and size of plant material, the composition of the culture medium used, the quality and quantity of light, and its duration affect the growth parameters, development and viability of cultured plant tissue. If the level of a factor is outside the normal tolerance range of a plant species, it will cause stress to it [2,3].

The excess water in the culture media is high compared to the normal soil conditions of plants. In the airspace of a closed culture vessel, the relative humidity can reach 95–99% [4]. This environment may cause developmental disorders, tissue and organ degeneration, like a poorly developed wax layer on the surface of plantlets and open stomata. Plant cells may absorb too much water, and organs become malformed and lose their

original functions. As a result, leaves and stems become bigger and swollen, often becoming translucent, a phenomenon called hyperhydricity. Callus production, the development of undifferentiated parenchymatic, highly vacuolated cells, also occurs. A high humidity environment reduces the evaporation of plant material, thereby modifying the absorption of ions from the culture medium. These processes decrease the plant quality and viability and may even lead to the death of the plant material [3,5]. Potato (*Solanum tuberosum* L. cv. Superior) plantlets in a hermetically sealed vessel showed the above-described symptoms in the study by Park et al. [6]. However, plantlets have grown healthy in a vented culture vessel, and also in a sealed culture vessel, when in the latter, potassium permanganate, as an ETH absorbent, was present [6].

CO_2 is also present in PTCs. In traditional tissue culture techniques, when sugar is present in tissue culture media, the plant metabolism is mixotrophic. Potato plantlets use both sugar and CO_2 as carbon sources in vitro, during the light period [7]. CO_2 level in the airspace of PTC varies according to the state of their photocycle, the type and growth state of the plant material and the actual sugar level in the culture media. Low levels of CO_2 (CO_2 starvation) reduced the growth of lily (*Lilium orinetalis* L. cv. Santander) and *Arabidpsis thaliana* L. Heynh. plantlets in vitro and resulted in an increased amount of reactive oxygen species (ROS) [2].

Accumulation of ETH in the PTC vessel deteriorates the quality of the plant, reduces its growth, regeneration potential and viability. Therefore, we need to eliminate it or limit its amount. To decrease or eliminate these harmful gaseous material, we need to ensure some aeration of tissue cultures, without the possibility of contamination [1,8].

In summary, in addition to other microenvironmental factors such as photoenvironment, temperature, or medium components, etc., it is necessary to control the ethylene, CO_2 levels and relative humidity in the airspace of culture vessels to reduce stress and ensure normal in vitro growth and development.

Although potatoes can be grown easily under tissue culture conditions in vitro using various explant types and several culturing techniques, it is also sensitive to culturing conditions, which may decrease the yield and regeneration potential of explants [9]. Potatoes, similar to other plants, are very sensitive to poorly ventilated culturing conditions. Chanemougasoundharam et al. [10] studied the difference between hermetic and non-hermetic closure types, applying aluminum foil, cotton plugs and plastic caps on culture tubes. Potato cultures covered with aluminum foil suffered from symptoms of poor ventilation, such as decreased leaf size, smaller shoot length with a bigger diameter (swollen shoots) and the presence of extra roots from shoots. Healthy development, higher chlorophyll content and dry mass were detected by using cotton plugs or plastic caps. Silver nitrate ($AgNO_3$) can regulate the activity of ethylene [11]. The use of $AgNO_3$ in the concentration of 2 mg L^{-1} in semi solid MS (Murashige and Skoog) medium [12] decreased the symptoms of excessive amounts of ethylene (epinasty, hyperhydricity) and showed the optimal stem length and bigger leaf area compared to the plantlets grown on a control MS medium without $AgNO_3$ [13]. Another study revealed the importance of aeration in potato in vitro micropropagation, when sealed cultures suffered from stress symptoms, like hyperhydration and reduction in growth parameters, but cultures with diffusive ventilation by semi-permeable plastic foil had less symptoms. Symptoms caused by poor aeration conditions can be decreased by the application of 3 μM $AgNO_3$ added to the culture medium. No symptoms were registered in the case of forced ventilation, even if 1-aminocyclopropane-1-carboxylic acid (ACC) (2 μM), an ethylene precursor, was added to the medium. In vitro tuberization was also considerably higher in the case of forced ventilation [14]. In the case of the multiplication of gloxinia (*Sinningia speciosa* Baill.) in vitro,

shoot regeneration tendency could be improved significantly by the application of ethylene inhibitors, such as silver thiosulphate (STS), aminoethoxyvinylglycin (AVG) and cobalt chloride [15].

Plant growth regulators (PGRs) have a very important function in plant response to environmental stresses. The level of abscisic acid (ABA) in plant tissues indicates the stress level of plants. It is proven that jasmonic acid (JA) and ETH play a key role in plant defense against environmental stress factors [16–19], but cytokinins, auxins, gibberellins and brassinosteroids are also involved in the response to abiotic stresses [20–22]. A novel study revealed that in vitro cultured potato plantlets were in serious stress when their level of ABA was much higher than in greenhouse-grown plants [23]. Salicylic acid (SA) is also an important signaling molecule in plant responses to various biotic and abiotic stressors. SA regulates the expression of stress-related genes in plant defense; thus, it helps to improve the plant's immunity to different stress factors. SA can also regulate the effect of other PGRs, especially JA and ETH via signaling pathways. Subsequently, SA helps to improve the yield of plant material (for, e.g., fruits) under stress conditions [24–26].

From the above studies, it can be concluded that aerating the vessels [10,11,14] or supplementing the medium with various chemicals [12,13,15] that cause a decrease in ethylene levels could effectively improve the regeneration, organogenesis, growth and quality of potato plantlets. Furthermore, other chemicals, such as various PGRs [20–22,24–26], which are involved in plant stress mitigation, can potentially be used to produce healthy, high-quality in vitro plantlets.

The aim of this study was to develop simple methods for producing high-quality in vitro potato (*Solanum tubersum* L. cv. Desirée) plantlets during both in vitro culture maintenance and propagation material production by reducing the potentially harmful effects of using tightly sealed culture vessels. To achieve this goal, the effects of aeration and some inorganic and organic chemical additives, including PGRs, on the quality and in vitro growth and developmental parameters of potato plantlets were investigated.

2. Materials and Methods

2.1. Plant Material and Experimental Design and Conditions

In vitro nodal cultures of potato (*Solanum tuberosum* L. cv. Désirée) were maintained on MS medium, complemented with 3% saccharose but without PGRs. The pH was set to 5.7–5.8 prior to autoclaving. Culture media were sterilized using an autoclave at 121 °C and 1.2 bar pressure for 20 min. All cultures were kept in a culture room at a temperature of 23 ± 2 °C and a 16/8 photoperiod provided via a 1:1 mix of daylight and warm white, fluorescent lamps in a vertical position, resulting in a light intensity of 80–106 μmol s^{-1} m^{-2}.

Plant multiplication was performed on a PGR-free medium using single-node segments to obtain sufficient plant material for the experiments. Each jar contained 5 nodal segments as explants on 70 mL of medium per jar, with a volume of 390 mL.

In the first experiments, different closure types of jars were tested. The various closures were as follows: (C1.) an intact, undrilled metal cap, without holes, i.e., control; (C2.) two layers of semi-permeable plastic foil; (C3.) a metal cap with only one hole; or (C4.) a metal cap with three holes. Metal caps were drilled with a drill bit (5 mm in diameter), and sponges were applied across the holes to avoid contamination and desiccation of the PTCs.

In the second experiments, additional chemicals were applied in the medium alone or in combination under tightly closed conditions, i.e., applying intact, airtight metal caps without holes (C1.). The applied chemicals were the following: (1) silver nitrate ($AgNO_3$), 2.0 mg L^{-1}, (2) 2.0 mg L^{-1} $AgNO_3$ in combination with NAA (1-naphtylacetic acid) (0.1 mg L^{-1}), (3) NAA, 0.1 mg L^{-1}, (4) MTP (meta-topoline; 6-(3-hydroxybenzylamino)purine), 0.1

mg L^{-1}, (5) VIT-C (ascorbic acid), 10.0 mg L^{-1}, (6) SA (salicylic acid), 0.1 mg L^{-1}, (7) JA (jasmonic acid), 0.1 mg L^{-1}, (8) GLU (glutamic acid), 0.3 mg l^{-1}. All of the additional chemicals were filter sterilized and added to the medium after autoclaving.

2.2. Data Collection and Statistical Analysis

Data were collected at the end of the subculture, after 4 weeks. Shoot number (SN), shoot length (SL) (mm) per explant, node number of shoots (NN), leaf size (LS) (mm^2) of the fourth apical leaves, root number (RN), root length (RL) (mm) of plantlets, shoot weight (SW) (mg), root weight (RW) (mm), and tuber number (TN) per jar were measured. Measurements were made from 10 jars and each jar contained 5 nodal segments as explants.

SPSS for Windows software (SPSS® version 21.0) was used for statistical analysis. One-way ANOVA followed by Duncan's test at $p < 0.05$ was applied to analyze the data of both experiments to compare the effect of different treatments.

3. Results

3.1. Effect of Culture Aeration

Different aeration of the cultures caused significant growth and developmental changes in plantlets (Table 1). Significantly, most shoots (SN: 1.3 ± 0.09) were produced in cultures covered with two layers of plastic foils (C2.) (Figure 1B). Other coverage methods were not significantly different from the control.

Figure 1. Effect of aeration of cultures on the growth and development of in vitro potato plantlets ((**A**): control; tightly closed coverage, (**B**): plastic foil coverage, (**C**): one-hole-caps coverage, (**D**): three-hole-caps coverage).

The SL was significantly higher in cultures covered with drilled caps (C3. and C4.) (Figure 1C,D) compared to closed cultures. It was significantly highest in cultures covered

with three-holed caps (C4.) (52.4 ± 2.6 mm) (Figure 1D). In cultures covered with one-holed caps (C3.) (Figure 1C), SL was significantly higher (42.4 ± 1.6 mm) than values of control cultures (23.1 ± 1.1 mm) (C1.). In cultures covered with plastic foil (C2.), SL was a bit lower than in totally closed control cultures, but the difference was not significant.

The difference in node numbers of regenerated shoots (NN) was small. NN was significantly the highest under closed conditions (7.4 ± 0.4) (C1.); however, it was the same in all of the drilled-capped cultures (C3. and C4.) but lower than in tightly closed jars. The plastic foil coverage (C2.) resulted in the lowest NN, but a statistically significant difference was proven only between the one-hole-capped (C3.) and plastic foil-covered (C2.) cultures.

Table 1. Mean values of measured parameters with standard errors in aeration experiment. Measurements are followed by letters that indicate significantly ($p < 0.05$) different values between treatments according to ANOVA and Duncan tests (abbreviations: SN: shoot number, SL: shoot length, NN: nodal number, LS: leaf size, RN: root number, RL: root length, SW: shoot weight, RW: root weight, TN: tuber number).

Type of Jar Closure	SN (pcs/shoot)	SL (mm/shoot)	NN (pcs/shoot)	LS (mm^2 of 4th apical leaf)	RN (pcs/plantlet)	RL (mm/plantlet)	SW (mg/jar)	RW (mg/jar)	TN (pcs/jar)
Tightly closed (C1.)	1.0 ± 0.02 b	23.1 ± 1.1 c	7.4 ± 0.4 a	0 b	2.8 ± 0.2 b	9.4 ± 1.0 b	44.2 ± 2.6 c	7.2 ± 21.5 c	0 b
Plastic foil coverage (C2.)	1.3 ± 0.08 a	19.6 ± 1.3 c	5.4 ± 0.4 c	2.9 ± 0.8 b	3.5 ± 0.4 b	18.6 ± 3.1 b	63.4 ± 4.5 c	16.3 ± 3.0 c	0.04 ± 0.06 b
One-hole-capped coverage (C3.)	1.0 ± 0.02 b	42.4 ± 1.6 b	6.5 ± 0.2 b	90.3 ± 6 a	7.0 ± 0.4 a	65.4 ± 3.6 a	206.0 ± 12.0 b	119.0 ± 10.5 b	0.3 ± 0.03 a
Three-hole-capped coverage (C4.)	1.1 ± 0.04 b	52.4 ± 2.6 a	6.0 ± 0.2 bc	95.0 ± 7.8 a	7.9 ± 0.4 a	71.9 ± 4.7 a	262.0 ± 18.2 a	204.0 ± 17.0 a	0.3 ± 0.06 a

The main effect of closed conditions was the development of strongly rudimentary leaf blades that cannot be measured practically (0 mm^2). Plastic foil coverage (C2.) had a similar inhibitory effect on leaf growth (LS: 2.9 ± 0.8 mm^2) as the undrilled, metal cap coverage (C1.). The effects of coverage with drilled caps (C3. and C4.) resulted in healthy leaf morphology and size. The highest leaf size was 95.0 ± 7.8 mm^2 in cultures covered with three-holed caps (C4.). However, the differences in LS between the cultures covered with the two types of drilled caps were very low and non-significant.

The number of roots per plantlet (RN) was significantly higher (7.0 ± 0.4 and 7.9 ± 0.4) in cultures covered with drilled caps (C3. and C4) in comparison with the RN of closed control cultures (C1.) (2.8 ± 0.2) or in plastic foil covered cultures (C2.) (3.5 ± 0.4). Numerically, the highest RN could be achieved by applying three-holed caps (C4 (7.9 ± 0.4). However, the number of holes on caps (C3. and C4) did not result in significantly different RNs.

Length of roots (RL) was also significantly the highest (65.4 ± 3.6 mm, 71.9 ± 4.7 mm) in cultures covered with drilled caps (C3. and C4.) compared with closed control cultures (C1.) (9.4 ± 1.0 mm) or with plastic foil covered (C2.) cultures (18.6 ± 3.1 mm).

SW also showed a positive correlation with aeration. The lowest SW could be measured in closed control cultures (C1.) (44.2 ± 2.6 mg). It was higher in value (63.4 ± 4.5) in cultures covered with plastic foil (C2.) but was statistically not significant from control cultures. Drilled-capped cultures (C3. and C4.) gave much higher SWs. It was 206 ± 12.0 mg per jar with one-holed caps (C3.) and 262.0 ± 18.2 mg per jar with three-holed caps (4.) were applied, respectively. SW was significantly the highest in response to the application of three-holed caps.

RW followed the same tendency as SW. The lowest RW was measured in closed culture conditions (C1.) (7.2 ± 21.5 mg). Root mass doubled in cultures covered with plastic foil (C2.), but the measured mean of RW was not significantly different from the mean RW

value of closed cultures. RW was closely related to the degree of aeration. Cultures resulted in 119.0 ± 10.5 mg root mass per jar when they were covered with one-holed caps (C3.) and 204.0 ± 17.0 mg per jar when three-holed caps (C4.) were applied. The last one, the three-holed cap coverage (C4.), resulted in significantly the highest root mass (RW).

No tubers were observed in closed control cultures (C1.). Cultures covered with one-holed caps (C3.) resulted in a few tubers but this was not significantly different from the control ones. Significantly, about tenfold higher amounts of tubers were produced in cultures covered with three-holed caps (C4.) or in cultures covered with plastic foil (C2.); the difference between the two covering methods was not significant.

3.2. Effects of Additional Chemical Additives in the Medium

In this part of our experiments, all cultures were under tightly closed conditions, covered with intact, undrilled caps (C1.). Applying additional chemicals in the medium significantly altered the growth and development of in vitro potato plantlets (Table 2).

Regarding the number of newly produced shoots (SN), the cytokinin (MTP) treated cultures (Figure 2G) gave the maximum SN value (1.9 ± 0.4). A high value was detected in response to the treatment of $AgNO_3$ applied in combination with NAA (1.6 ± 0.1) (Figure 2C) and to the JA treatment (1.6 ± 0.1) (Figure 2E), respectively. MTP, $AgNO_3$ + NAA or JA resulted in significantly higher amounts of shoots than were observed in the control cultures (1.2 ± 0.06) (Figure 2A). All other additional chemicals resulted in a similar SN to the control one.

The length of shoots (SL) was the highest from the impact of JA (29.2 ± 1.1 mm) (Figure 2E), and it was the same from the impact of NAA alone (28.8 ± 1.0 mm) (Figure 2D) or GSH (26.4 ± 0.8 mm) (Figure 2H). The differences between them were not significant, but all of them were significantly higher compared to the values of control cultures (23.2 ± 1.1). $AgNO_3$, MTP or SA treatment (Figure 2B, G, I) resulted in significantly lower SL than the control treatment, but the treatment of silver nitrate led to the lowest SL (13.2 ± 0.9).

The NNs of regenerated shoots were the highest after JA treatment (11.5 ± 0.6) (Figure 2E). High NNs were detected after NAA treatment (Figure 2D) as well. GSH treatment (Figure 2H) resulted in the same NNs as the control one (7.4 ± 0.4) (Figure 2A). All other treatments resulted in significantly lower NNs, but the treatment with $AgNO_3$ was the lowest among them (3.0 ± 0.2) (Figure 2B).

$AgNO_3$ had the greatest effect on LS (leaf blade size). The highest leaf size resulted from the $AgNO_3$ treatment when it was applied alone (12.1 ± 3.0 mm^2) (Figure 2B). $AgNO_3$applied in combination with NAA (Figure 2C), resulted in only half the dimension of leaf size (6.3 ± 2.2 mm^2), but this value was also significantly different from the values of untreated (control) cultures (Figure 2A), where practically no leaf blades were measurable.

JA treatment (Figure 2E) yielded the highest number of roots (RN) (11.7 ± 0.7), but the treatment with NAA (Figure 2D) resulted in high amounts of roots (10.7 ± 0.6) as well. NAA applied in combination with $AgNO_3$ (Figure 2C), also stimulated root production but in a much lower degree than NAA alone (5.5 ± 0.7). GSH (Figure 2H). Similarly to NAA, in combination with $AgNO_3$, it also increased the RN, albeit to a lesser extent, but it was significantly higher than the value measured in control cultures (2.8 ± 0.2) (Figure 2A).

The length of roots (RL) was notably the highest in cultures containing $AgNO_3$ applied in combination with NAA (33.5 ± 4.9 mm) (Figure 2C); however, NAA treatment alone (Figure 2D) resulted in the same value. JA treatment (Figure 2E) also elevated the length of roots significantly (20.8 ± 1.0) in comparison with the treatment of GSH. The treatment of GSH (Figure 2H) also resulted in moderately but significantly higher RL (16.9 ± 1.3) than the control cultures (9.4 ± 1.0) (Figure 2A).

Figure 2. Effects of additional chemicals on the growth and development of in vitro potato plantlets ((**A**): control, i.e.,: no additional additives, (**B**): $AgNO_3$ treatment, (**C**): combination of $AgNO_3$ and NAA, (**D**): NAA treatment, (**E**): JA treatment, (**F**): vitamin C treatment, (**G**): MTP treatment, (**H**): GSH treatment, (**I**): SA treatment).

Table 2. Mean values of measured parameters with standard errors of the shoot treatment experiment. Measurements are followed by letters that indicate significantly ($p < 0.05$) different values between treatments according to ANOVA and Duncan tests (abbreviations: $AgNO_3$ + NAA: silver nitrate, in combination with NAA (1-Naphthaleneacetic acid), NAA: 1-Naphthaleneacetic acid, JA: jasmonic acid, SA: salicylic acid, $AgNO_3$: silver nitrate, MTP: meta-topoline, GSH: glutathione, SN: shoot number, SL: shoot length, NN: nodal number, LS: leaf size, RN: root number, RL: root length, SW: shoot weight, RW: root weight, TN: tuber number).

Chemical Additives	SN (pcs/shoot)	SL (mm/shoot)	NN (pcs/shoot)	LS (mm^2 of 4th apical leaf)	RN (pcs/plantlet)	RL (mm/plantlet)	SW (mg/jar)	RW (mg/jar)	TN (pcs/jar)
no additives	1 ± 0.2 c	23.2 ± 1.1 b	7.4 ± 0.4 b	0 c	2.8 ± 0.2 d	9.4 ± 1.0 d,e	44.2 ± 2.6 e	7.2 ± 1.5 d	0 c
$AgNO_3$ + NAA	1.6 ± 0.09 a,b	21.1 ± 0.09 b,c	4.0 ± 0.3 d	6.3 ± 2.2 b	5.5 ± 0.7 b	33.5 ± 4.9 a	90.6 ± 9.3 b	47.9 ± 10.2 c	0.3 ± 0.07 a
NAA	1.3 ± 0.07 b,c	28.8 ± 1.0 a	10.4 ± 0.5 a	0 c	10.7 ± 0.6 a	33.3 ± 2.0 a	124.6 ± 7.0 a	123.3 ± 8.0 a	0 c
JA	1.6 ± 0.1 a,b	29.2 ± 1.1 a	11.5 ± 0.6 a	0 c	11.7 ± 0.7 a	20.8 ± 1.0 b	139.4 ± 7.7 a	97.2 ± 7.3 b	0 c
SA	1.0 ± 0.02 c	15.0 ± 1.2 d	5.2 ± 0.05 c	0 c	2.7 ± 0.5 d	4.9 ± 0.9 e	42.5 ± 3.3 e	2.0 ± 0.7 d	0.02 ± 0.02 c
$AgNO_3$	1.2 ± 0.06 b,c	13.2 ± 0.9 d	3.0 ± 0.2 d	12.1 ± 3 a	2.3 ± 0.3 d	13.2 ± 1.7 c,d	55.5 ± 5.8 d,e	5.1 ± 1.0 d	0.1 ± 0.05 b
VIT-C	0.9 ± 0.05 c	20.0 ± 1.5 b,c	5.9 ± 0.4 c	0 c	3.6 ± 0.4 c,d	8.2 ± 1.0 d,e	43.4 ± 4.0 e	9.4 ± 1.6 d	0 c
MTP	1.9 ± 0.4 a	18.5 ± 0.7 c	5.5 ± 0.2 c	1.2 ± 0.2 c	2.2 ± 0.3 d	7.9 ± 1.4 d,e	72.9 ± 5.2 c	7.1 ± 1.5 d	0 c
GSH	1.0 ± 0.02 c	26.4 ± 0.8 a	8.3 ± 0.3 b	0 c	4.9 ± 0.3 b,c	16.9 ± 1.3 b,c	70.9 ± 3.8 c,d	17.5 ± 2.3 d	0 c

Treatment with JA (Figure 2E) resulted in the highest shoot mass (SW) (139.4 ± 7.7 mg), but treatment with NAA alone (Figure 2D) gave similar results (124.6 ± 7.0). NAA applied in combination with $AgNO_3$ (Figure 2C), caused a lower value (90.6 ± 9.3) than previous treatments. The treatment with MTP or GSH (Figure 2G-H) gave the same results (72.9 ± 5.2, 70.9 ± 3.8), and they caused a higher SW than was detected in control cultures (9.4 ± 1.0) (Figure 2A).

The weight of the roots (RW) was significantly the highest in cultures treated with NAA alone (123.3 ± 8.0 mg) (Figure 2D). JA (Figure 2E) strongly increased the RW (97.2 ± 7.3 mg) as well. RW was also considerably high in cultures treated with a combination of NAA and $AgNO_3$ (47.9 ± 10.2 mg) (Figure 2C), but it was less than half of the result produced with NAA alone. Only GSH (Figure 2H) enlarged the RW to a higher degree, but the difference from values of control cultures or from the results of other treatments was not significant.

The highest TN was observed in the presence of $AgNO_3$, in combination with NAA (0.3 ± 0.07) (Figure 2C), but tubers were also produced in the presence of $AgNO_3$ alone (0.1 ± 0.05) (Figure 2B). The latest was also significantly higher than in control cultures (Figure 2A), where the tuber number was zero. SA (Figure 2I) stimulated the production of tubers, but to a very small extent (0.02 ± 0.02). No tubers developed in response to any other treatments.

4. Discussion

4.1. Effect of Culture Aeration

The average SN was significantly higher than one in the case of plastic foil coverage, but other treatments did not modify it. This may be explained due to the partial permeability of the plastic foil, resulting in different air compositions in the culture vessels compared with other treatments.

The average SL was much higher in cultures covered with drilled caps in comparison with the results of plastic foil or undrilled caps coverage. Plantlets developed in tightly closed cultures showed slight signs of hyperhydricity and developmental disorders, such as slightly swollen stems, leaf degeneration, fewer and shorter roots, and callus production (Figure 1A). These results correlate with earlier findings [14], when increasing the ventilation of vessels improved the in vitro growth of potatoes, increased stem length, reduced hyperhydricity, and leaf epinasty did not occur. This phenomenon may be explained by faster removal of the ethylene produced by the plant from the culture container in response to increased aeration [14,27,28]. The higher NN counted in closed cultures can presumably be due to the presumed accumulation of ethylene in culture vessels, and also correlates with the literature [10]. In the relevant study by Chanemougasoundharam et al. [10], the type of closure affected the number of leaves in a genotype-dependent manner; however, when the aeration was increased due to the closure type, the number of leaves increased as well. The ethylene accumulation in closed culture vessels spectacularly affected the growth and size of the leaf surface. Leaves could not be developed well, or their growth was rudimentary, with no visible leaf surface in cultures capped with tightly closed, undrilled caps. Increased aeration likely led to reduced ethylene and humidity levels in the airspace of the jars, leading to healthier growing leaves with a larger leaf area. This finding was consistent with the studies of others [14,27], as diffusive and forced aeration resulted in a 3–5-fold increase in leaf area in potato in vitro cultures [14], while leaf expansion was reduced by about half in an in vitro culture of *Ficus lyrata* Warb. in closed cultures compared to ventilated ones [27]. The average number of roots (RN) correlated with the aeration level of vessel closure and accordingly with the presumed level of ethylene [14]. The minimum number of roots was formed in tightly closed cultures. Only a little bit more roots were developed in the case of plastic foil coverage compared to tightly closed control cultures. Drilled cap coverages resulted in the highest RN [29]. The number of roots was proportional to the number of holes in drilled caps.

The presumably accumulated ethylene in culture vessels highly affected the average length of roots (RL). It was the shortest in the case of tightly closed culture vessels. Plastic foil coverage resulted in longer roots than the control did. However, it was significantly higher in cultures covered with drilled caps and was also proportional to the number of drilled holes. The inhibitory effect of accumulated ethylene on root growth was also detected by others [14,29]. Both diffusive and forced ventilation increased the root length of in vitro potato plantlets 3–5-fold, respectively [14]. Root elongation is regulated by crosstalk between ethylene and auxin, as was demonstrated in *Arabidopsis thaliana*, and high levels of ethylene can inhibit it [29].

The ethylene, presumably accumulated in tightly closed culture vessels, negatively affected the shoot growth; therefore, the shoot mass (SW) was also the lowest in this case. The plastic foil coverage resulted in a slight increase in SW, which can be explained by the partial permeability of the plastic foil. The differences were also the greatest in the case of drilled cap coverage; SW was multiple times higher compared to closed control cultures, and was proportional to the number of drilled holes [27]. The average RW showed the same tendency as it did in SW.

The presumed accumulation of ethylene in culture vessels due to their closure obviously blocked the in vitro tuber development as well [14,30]. Accordingly, cultures covered with plastic foil or with three-holed caps showed higher TN.

In summary, aeration, especially the use of drilled caps, improved the quality and development of potato seedlings. Ventilation had a dual effect, contributing to the removal of excess water, thereby reducing the relative humidity, and removing ethylene from the airspace of the jars.

4.2. Effects of Additional Chemical Additives in the Medium

Of the chemicals applied in culture media, MTP increased the number of newly developed shoots (SN) to the greatest extent. This is not surprising because MTP is a type of cytokinin and, thus, can promote the formation of new shoots [31]. NAA applied in combination with $AgNO_3$ or JA, also promoted the development of new shoots, but to a lesser extent than MTP. The shoot-induction promoting effect of JA is well-known [32]. JA also promoted shoot branching. The presence of silver ions can decrease not only the ethylene production [33], but also the sensitivity of ethylene receptors to ethylene, because silver ions bind to the ethylene receptors irreversibly displacing copper ions [34]. NAA, a kind of auxin, can also modulate ethylene production and may help to mitigate the abiotic and biotic stresses [21] Furthermore, it may promote the growth of various plant parts, as it enhances stem length, leaf area, root elongation, etc. [35]. The growth promotion of NAA and the ethylene inhibitory effect of silver ions may interact synergistically to affect the number of newly developed shoots (SN). Single applications of NAA or $AgNO_3$ could enhance the SN, but to a lesser extent (Table 2).

The SL was the highest on the medium containing JA or NAA, but they were deformed, and their leaves were rudimentary (Figure 2D,E). JA has a complex effect; it may promote both the shoot and root growth, and it has a role in the defense against abiotic and biotic stresses [18]. GSH also promoted SL, but to a lesser extent. Glutathione promotes cell proliferation and controls cell regeneration via the GSH–GSSG redox pair system and helps to mitigate the stress reaction of plants [36]. The presence of silver ions shortened the SL compared to the control cultures. It may be a result of the decreased sensitivity of shoot cells to ethylene caused by silver ions [34], considering that a high amount of ethylene induces the extra elongation of shoots [37]. While MTP promoted the growth of new shoots, in parallel, it decreased the length of the shoots. SA or VIT-C also decreased the SL, to a lesser extent, presumably due to their stress-mitigating effects when the putatively increased ethylene level had caused an abiotic stress [26,38].

The number of nodes (NN) was also the highest when culture media contained JA or NAA. These additives also promoted the development of new nodes. The elongation of shoots by JA has a shoot-induction-promoting effect [21,32]. The presence of GSH in culture medium also increased the NN, but to a lesser extent. The presence of silver ions in culture medium decreased the NN; this may also be in connection with the decreased sensitivity of shoot cells to ethylene by silver ions [37]. SA, MTP or VIT-C decreased the number of nodes similarly, as well as the SL.

Silver ions had an obvious effect on the development of leaves, especially on the size of the leaf surface. On media, which contained silver ions, healthy leaves were developed, whereas in the presence of both NAA and $AgNO_3$, there were fewer leaves with a smaller surface. In control cultures, only immature leaves were developed with no measurable surfaces, which is supposedly due to the presence of ethylene and its accumulation in closed cultures [13].

The number of roots (RN) was significantly increased in the cultures that contained JA or NAA. NAA is an auxin-type PGR; thus, it stimulates the development of roots [39]. JA, together with ethylene and auxins, controls the root development [40,41]. NAA, in

combination with $AgNO_3$, resulted in a lower root number, while $AgNO_3$, when applied alone, had no effect on the root number. GSH increased the root number to a lesser extent. This may be caused by the cell proliferation, growth promotion and stress mitigation capability of GSH [36].

The root length (RL) was the highest in the presence of NAA when applied in combination with $AgNO_3$, but the difference was not significant when NAA was applied alone. JA increased the RL only to a lesser extent. GSH elevated the root elongation to a lesser extent. $AgNO_3$, applied alone, also promoted root elongation, which may be in connection with the decreased sensitivity of shoots to ethylene caused by silver ions [29].

JA elevated the SW to the greatest extent, which was followed by NAA treatment. JA can stimulate the expansion of potato tuber cells by increasing the water uptake and may also be in other cells of the potato plants [42]. NAA applied in combination with $AgNO_3$, also elevated it, but to a lesser extent compared to the former treatments.

The RW increased most significantly from NAA, having an auxin effect, followed by JA. The presence of $AgNO_3$ also increased it.

Higher amounts of tubers were produced when NAA was combined with $AgNO_3$. It may be assigned both to the ethylene-action-inhibitory effect of silver ions and to the growth-promoting effect of NAA. Tuber production increased only to a lesser extent in the presence of $AgNO_3$ alone. SA also increased it to a lesser extent, in accordance with earlier studies on its effect on tuber production [43]. SA also has an ethylene inhibitory effect [44]. No tuber was formed on the control medium because the presumed ethylene accumulation in the cultures inhibited its development [30]. JA treatment was also able to enhance tuber production in earlier studies [45], but in our present experiments, this effect could not be detected.

In summary, the growth and development, as well as the quality, of potato plantlets in tightly sealed culture jars can be improved by the application of $AgNO_3$ alone or in combination with NAA. The former can be recommended in the subcultures of the propagation phase, and the latter in the last subculture before acclimatization, as the combined application of $AgNO_3$ and NAA improved rooting.

5. Conclusions

The harmful effects of ethylene, which presumably accumulates in tightly sealed jars, on potato plant development and growth were prevented in our experiments in two ways: either by ventilating the lids using one- or three-holed caps or by adding chemicals, like $AgNO_3$ or the combination of NAA and $AgNO_3$, to the culture medium. Increased aeration of in vitro potato cultures improved the quality of plant material compared to control cultures covered with undrilled, tightly closed caps. Passive aeration through caps containing three holes with a sponge proved to be significantly the best for all of the measured morpho-physiological parameters, especially the leaf size, and resulted in well-developed, healthy shoots. One-holed caps coverage may also be preferable, because the difference between the effects of one- and three-holed caps on the majority of morpho-physiological parameters of in vitro potato plantlets was not significant. Applying plastic foil caused fewer differences in measured parameters compared to the control. The effect of passive aeration using drilled caps was presumably doubled; it may have contributed to reducing the humidity in the jars, and it also facilitated the removal of excess ethylene from the airspace of the jars.

Under tightly closed culture conditions, leaf development was the best when silver ions in the form of $AgNO_3$ was present in the culture medium alone (without any other additives), resulting in a well measurable leaf size (12.1 ± 3 mm^2), while the value of the root number (2.3 ± 0.3) was below that of the control (2.8 ± 0.2), and the root length (13.2 ± 1.7 mm) was also remained low (control was 9.4 ± 1.0 mm). Shoot length (13.2 ± 0.9 mm)

was also under the level of the control (23.2 ± 1.1 mm). Silver ions, combined with NAA, also elevated the number of developed leaves, but to a lesser extent than it was in the previous treatment. However, they resulted in higher root number (5.5 ± 0.7) and the highest root length (33.5 ± 4.9 mm) compared to the results of control cultures (RN: 2.8 ± 0.2, RL: 9.4 ± 1.0 mm).

The current experiments allowed for a comprehensive comparison of the effects of simple types of culture aeration with the use of various chemicals, including potentially effective PGRs, to improve the quality and development of in vitro potato plantlets. The effects of these factors on in vitro plants have not been investigated so widely, with only a few factors being compared in a single experiment. As a result, this study describes very simple methods for improving the quality, growth and development of in vitro potato plantlets under various culture conditions. They are cost-effective and widely applicable, as they are based on either passive aeration or the application of very simple chemicals in the culture medium. Passive aeration with drilled caps was able to eliminate excess water and ethylene from the airspace of the jars, while the addition of $AgNO_3$ alone, or in combination with NAA, was able to reduce the effect of excessive ethylene. We can conclude that, if possible, using culture vessels with one- or three-holed caps is the most recommended way to produce high-quality in vitro potato plantlets because the aeration had a greater effect on the plant development and quality than chemicals used in the culture media. When plant material is cultured under tightly closed lids, the application of additional chemical substances is recommended. The application of silver ions in the culture media of potato plantlets is preferable for culture maintenance. However, for acclimation, the combination of silver ions and NAA seems to be the best due to its higher root induction capability.

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Abbreviations

The following abbreviations are used in this manuscript:

PTC	Plant tissue culture
CO2	Carbon dioxide
ROS	Reactive oxygen species
ETH	Ethylene
$AgNO_3$	Silver nitrate
ACC	1-aminocyclopropane-1-carboxylic acid
STS	Silver thiosulphate
AVG	Aminoethoxyvinylglycin
PGR	Plant growth regulator
NAA	1-Naphthaleneacetic acid
ABA	Abscisic acid
JA	Jasmonic acid
SA	Salicylic acid
MTP	Meta-topoline (6-(3-hydroxybenzylamino)purine)

GSH	Glutathione
VIT-C	Ascorbic acid

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