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Production of Virus-Free Garlic Plants through Somatic Embryogenesis

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Abstract: The present study was conducted to establish a protocol for the regeneration of virus-free garlic plants through somatic embryogenesis of two Croatian garlic ecotypes. Basal parts of cloves from mother plants were cultured on a full Murashige and Skoog (MS) or modified MS medium ($\frac{1}{4}$ of KNO_3 and NH_4NO_3 and 2x$MgSO_4$) containing 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) or 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin (Kin) and representing four different treatments. Plants were regenerated in MS medium containing 0.1 mg L^{-1} 2,4-D and rooted in a medium containing 0.05 mg L^{-1} 1-naphthaleneacetic acid (NAA) + 0.005 mg L^{-1} 6-(γ,γ-dimethylallylamino)purine (2iP). The presence of viruses (i.e., sanitary status) of the mother plants and regenerants was checked by enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). The mother plants were infected with onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV). In addition, the presence of garlic common latent virus (GCLV) was confirmed in four mother plants. Embryogenic callus developed in all four treatments with success ranging from 55% to 81% depending on treatment and ecotype. Plant conversion was significantly higher in somatic embryos developed in media containing 0.1 mg L^{-1} 2,4-D than those developed in media containing 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin. Virus elimination success ranged from 13.3% up to 62.5% depending on garlic ecotype and treatment. The overall rate of virus elimination by somatic embryogenesis for both treatments and ecotypes were 20.7%, 22.9%, and 30.5% for OYDV, GCLV, and LYSV, respectively. Based on these results, somatic embryogenesis has been shown to be equally or more successful in eliminating garlic viruses compared to other in vitro methods.

Keywords: *Allium sativum*; callogenesis; somatic embryos; virus elimination

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1. Introduction

Garlic (*Allium sativum* L.) is one of the oldest known crops grown worldwide, reaching an annual production of 30.708 million tonnes [1]. The well-known beneficial effects of garlic on human health are the main reason for its presence in global diet habits, and they are mainly attributed to allicin and other organosulfur compounds present in garlic bulbs [2].

Garlic varieties and landraces do not produce fertile flowers or seeds; therefore, they are propagated vegetatively by planting cloves or aerial bulbils. In Croatia, garlic is grown mainly in small family farms for local markets and home consumption with relatively low yields due to the poor quality of planting material and inadequate storage conditions [3,4]. Growers usually save a certain amount of yield for planting in the next season. In this way, they secure their own propagation material and also maintain biodiversity. Therefore, high genetic and phenotypic variability is present in a relatively narrow area.

Viral diseases can play a significant role in agricultural crop production, especially in vegetatively propagated plants. Because viral infections are usually systemic, they occur in organs such as tubers, rhizomes, stolons, bulbs, and buds used for vegetative propagation, which, in the case of garlic, contributes to the accumulation and spread of viral diseases through infected bulbs. To date, more than 15 garlic viruses from different genera (mainly *Potyvirus*, *Carlavirus*, *Allexvirus*) transmitted by vegetative propagation and various vectors have been reported with significant impacts on garlic production [5]. As previously reported by Vončina et al. [6] and Vončina et al. [7], garlic ecotypes grown in Croatia showed a deteriorated sanitary status, especially in terms of infection rates with onion yellow dwarf virus (OYDV)—98.7%, garlic common latent virus (GCLV)—49.4% and leek yellow stripe virus (LYSV)—40.5% with only 1.3% virus-free plants. Recently, part of the garlic collection maintained under the National Programme for the Conservation and Sustainable Use of Plant Genetic Resources was tested for the presence of viruses [8]. The study confirmed the presence of GCLV, LYSV, OYDV and shallot latent virus (SLV) in the collection. In addition, 78% of the cloves tested were found to have a mixed infection with all four viruses.

Viral infections reduce garlic bulb weight up to 60% [9,10]. Conci et al. [5] found that after virus-free plants were planted in the field, yields were expected to be significantly reduced in subsequent years of cultivation due to virus exposure and reinfection. However, after 5 years of cultivation in the field, yields were still higher than those of chronically infected plants. This suggests that periodic renewal of 'seed cloves´ with new virus-free material is necessary to maintain the profitability of the crop [5].

The production of virus-free garlic plants through traditional agronomic systems is difficult to implement, and expensive because it must be done in an area free of viral vectors [11] once virus-free plants are available. To overcome these barriers, in vitro propagation of virus-free plants can be used to obtain a large number of virus-free plants in a short time [12].

The most commonly used method for virus elimination from garlic is shoot meristem culture alone [13], in combination with solar heat treatment [11] or hot air treatment [11,14,15]. Kudělková et al. [16] successfully eliminated GCLV in five garlic genotypes combining shoot meristem culture and chemotherapy with ribavirin. Senula et al. [17] found that the addition of ribavirin to the induction medium increased virus elimination, but reduced plantlet regeneration. The efficiency of ribavirin, but also its phytotoxicity was reported by Danci et al. [18]. Instead of shoot meristem culture, Haque et al. [19] used root tip culture followed by the separation of regenerated tiny shoots from the mother explant (two-step process) for virus elimination from garlic. Somatic embryogenesis alone or combined with thermotherapy [20–22] was effective in eliminating grapevine viruses. Somatic embryogenesis has also been successfully used for virus elimination in species such as cacao [23], cassava [24], and sugarcane [25].

Plant regeneration by somatic embryogenesis is a rare phenomenon in garlic [26]. Optimization of media composition is necessary to ensure better yield and quality of somatic embryos in garlic [27]. Moreover, successful regeneration depends on the genotype, the type of explant and its physiological conditions, and the combination of growth regulators in the medium [28]. Several reports have presented embryogenesis in garlic tissue culture [26,29–33] by optimising the media components and the type of explant. To the best of our knowledge, there are no reports on the regeneration of virus-free garlic plants by somatic embryogenesis. Therefore, the aim of this study was to test the effect

of different media compositions on (1) the success of plant regeneration through somatic embryogenesis of two Croatian garlic ecotypes and (2) production of virus-free garlic plants through somatic embryogenesis.

2. Materials and Methods

2.1. Plant Material

Two ecotypes of garlic were obtained from the Croatian garlic collection of the Institute of Agriculture and Tourism Poreč, which is maintained within the National Programme for the Conservation and Sustainable Use of Plant Genetic Resources for Food and Agriculture. The ecotype 'Istarski crveni' (IPT013) is listed as a conservation variety on the Variety List of the Republic of Croatia, while the ecotype IPT012 is also grown by local producers in the Istria region, but is not registered as a variety. Both ecotypes are bolting with red-coloured cloves.

2.2. Culture Establishment, Induction of Somatic Embryogenesis and Plant Regeneration

The garlic bulbs selected for this experiment were of average size for ecotype and without symptoms of disease, physiological or mechanical damage. Cloves from eight bulbs of garlic of each ecotype were peeled off from the outer skin and treated separately for each bulb. The cloves were washed under running tap water for 15 min and then disinfected in 70% ethanol for 1 min, followed by 15 min of shaking in a 5% solution of sodium dichloroisocyanurate dihydrate (Izosan® G, Pliva, Zagreb, Croatia) supplemented with 3 drops 100 mL^{-1} of surfactant Tween 20. Finally, the cloves were washed three times for 3 min in sterile distilled water. A two-millimetre thick basal part portion of each clove was cut off and divided into four equal parts. These parts were considered explants. Six such explants of each bulb were then placed in 60 mm Petri dishes in four different medium compositions/treatments (Table 1). This resulted in six explants/bulbs/treatments giving a total of 48 explants per ecotype x treatment combination (192 explants per ecotype). The explants, later, the regenerated plants from each bulb were cultured separately and recorded accurately throughout the experiment, as we did not know in advance the health status of the mother plants, i.e., the presence of the virus.

Table 1. Composition of media used for induction of callogenesis and somatic embryogenesis.

Treatments	Composition
MS2,4-D	MS salts and vitamins (Murashige and Skoog [34]), myoinositol 0.1 g L^{-1}, sucrose 30 g L^{-1}, proline 100 mg L^{-1}, casein hydrolysate 100 mg L^{-1}, 2,4-D 0.1 mg L^{-1}, Plant agar (Duchefa) 6 g L^{-1}; pH 5.8
MS2,4-D + Kin	MS salts and vitamins, myoinositol 0.1 g L^{-1}, sucrose 30 g L^{-1}, proline 100 mg L^{-1}, casein hydrolysate 100 mg L^{-1}, 2,4-D 1 mg L^{-1}, Kin 0.5 mg L^{-1}, Plant agar (Duchefa) 6 g L^{-1}; pH 5.8
modMS2,4-D	Same as MS2,4-D but with $\frac{1}{4}$ of KNO_3 and NH_4NO_3 and 2x$MgSO_4$ as compared with full strength MS
modMS2,4-D + Kin	Same as MS2,4-D + Kin but with $\frac{1}{4}$ of KNO_3 and NH_4NO_3 and 2x$MgSO_4$ as compared with full strength MS

The culture was incubated in the dark at 24 °C for 25 days. At this time, callogenesis and the occurrence of somatic embryogenesis were assessed. The presence of early stages of somatic embryos was analysed under a stereomicroscope (Optica SZM-LED2, Italy). Explants were then subcultured on the same medium and further incubated at a light

intensity of 45 μE m^{-2} s^{-1} and a photoperiod of 16/8 days/nights. Since there was no evidence of regeneration (plant conversion) despite the presence of embryogenesis, callus induced on MS2,4-D + Kin and modMS2,4-D + Kin were subcultured on MS2,4-D treatment on day 35 after experimental setup. These treatments were designed as MS2,4-D + Kin→MS2,4-D and modMS2,4-D + Kin→MS2,4-D.

Embryogenesis efficiency was expressed as the percentage of explants that developed somatic embryos, and plant regeneration as the number of regenerated plants per embryogenic callus.

2.3. Rooting and Acclimatisation

For rooting, MS medium supplemented with 0.05 mg L^{-1} NAA + 0.005 mg L^{-1} 2iP and 8 g L^{-1} Bacto Agar (Difco Bacto) was used. The regenerated plantlets were transferred to the rooting medium from the 40th day after the start of the experiment for the next four months. In the analysis of rooting success, there was no difference in pre-treatments between the full and modified medium, but only in terms of hormone content during somatic embryogenesis/plant regeneration (MS2,4-D or MS2,4-D + Kin→2,4-D treatments). Thus, plants of the same ecotype, bulb, and treatment (full or modified) were transplanted into the same Magenta vessels in the rooting medium.

Plants with a developed root were transplanted into a peat substrate and irrigated with a 0.15% solution of the fungicide Previcur Energy (Bayer, Leverkusen, Germany), covered with a transparent plastic cover, and acclimatised at 18 °C in a growth chamber. After acclimatisation, the plants were grown outdoors under the net.

2.4. Virus Detection and Efficiency of Virus Elimination

The presence of viruses (i.e., sanitary status) of the mother plants was determined simultaneously with the presence of viruses in the regenerated plants. For this purpose, two cloves were planted from each mother plant, and the plants were grown four months before leaf tissue was collected for analysis. Regenerated, acclimatised plants were grown outdoors under a net for two months before leaf samples were taken for laboratory analysis. The sanitary status of the mother plants and regenerants were tested by ELISA and reverse transcription-polymerase chain reaction (RT-PCR) as a confirmation test of the ELISA results.

2.4.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Mother plants and their regenerants obtained through somatic embryogenesis were tested for four viruses by ELISA: OYDV, LYSV, GCLV and SLV. The ELISA kits were provided by Bioreba AG (Kanton Reinach, Switzerland) and the tests were performed according to the manufacturer's instructions using leaves as a potential antigen source. ELISA screening was performed on a total of 90 samples: 8 mother plants and 82 plants produced through somatic embryogenesis (64 of IPT013 and 18 of IPT012). Samples with an OD value at least 2 times greater than the average value of the negative controls were considered positive for a particular virus.

2.4.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In addition to the ELISA negative results and to verify the sanitary status at the end of vegetation, the cloves of the plants produced by somatic embryogenesis from virus-infected mother plants were additionally tested by RT-PCR using corresponding mother plants as positive controls. Cloves from the same plant were homogenised and 0.1 g of the extract was used for total RNA isolation. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and the concentration and quality of RNA were determined spectrophotometrically using P300 NanoPhotometer (Implen, München, Germany). RT-PCR was performed using primers described by Fidan and Baloglu [35] for GCLV, while primers for OYDV and LYSV were described by Dovas et al. [36], resulting in amplicons of 481, 283 and 304, respectively. RT-PCR was not performed for SLV because this virus

was not detected in any plant by ELISA. For RT-PCR, a one-step RT-PCR kit (Qiagen, Valenzia, CA, USA) was used in a reaction volume of 10 µL, consisting of 2 µL of 5xQiagen one-step buffer, 2 µL of Q solution, 0.4 µL of dNTP mix, 0.4 µL of enzyme mix, 3.8 µL of RNase-free water, 0.4 µL of each primer (10 µM) and 1 µL of sample RNA (approximately 30 ng). Cycling conditions were as follows: reverse transcription at 50 °C for 50 min, initial denaturation at 95 °C for 15 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 30 s; followed by a final elongation at 72 °C for 10 min. Products were analysed by electrophoresis on a 1.5% agarose gel prepared using GelRed (Biotium, Fremont, CA, USA) in 1XTBE buffer and visualised on the UV Transilluminator 2000 (Bio-Rad, Hercules, CA, USA).

2.5. Statistical Analysis

All experiments were set up in a completely randomised design. The effects of treatments and ecotypes in somatic embryogenesis, regeneration efficiency, and rooting were determined using the general linear model, two-way analysis of variance (ANOVA). Bonferroni tests at $p \leq 0.05$ were performed for mean comparisons. Statistical analysis of the data was carried out using the SAS/STAT® [37] programme package.

3. Results and discussion

3.1. Culture Establishment, Callus Formation and Induction of Somatic Embryogenesis

All explants of ecotype IPT013 were successfully established, while sporadic fungal infections occurred on the explants of ecotype IPT012, due to which about 15% of explants were discarded. Callus formation was 100% effective regardless of the media composition. This is in agreement with the experiment of Luciani et al. [38] who found that the production of garlic callus in a medium supplemented with 0.45–4.5 µM 2,4-D is maximal (100%) regardless of the presence or absence of cytokinin 6-benzylaminopurine (BAP). Haque et al. [39] also obtained very high levels of callogenesis using garlic root tip explants and similar concentrations of 2,4-D (0.5–5 µM). In this experiment, treatments with a combination of 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin (MS2,4-D + Kin) led to the production of slightly larger calli (visually estimated) than treatments with 0.1 mg L^{-1} 2,4-D (MS2,4-D). In addition to the small size difference, explants incubated on MS2,4-D developed long roots on calli, which were cut off during the first subculture. No difference in callus formation was observed between the full and modified MS medium (with reduced nitrate content). However, Sata et al. [32], found that the White medium [40], containing lower nitrate content and almost twice the concentration of $MgSO_4$, is more suitable for the induction of somatic embryogenesis from the basal parts of the garlic clove compared with the MS medium.

The percentage of embryogenic callus that developed ranged from 55% for IPT012 on modMS2,4-D + Kin to 81% for IPT013 on MS2,4-D and was not significantly affected by ecotype, treatments, or their interaction (Figure 1). Although non-significant, generally slightly lower embryogenesis was obtained with the MS2,4-D + Kin and modMS2,4-D + Kin treatments compared to the MS2,4-D treatment. Sata et al. [32] obtained the highest embryogenesis in the medium containing 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin. Although the presence of auxins is crucial for embryo initiation [32,41,42], the concentration of 1 mg L^{-1} 2,4-D seems to negatively affect the development of somatic embryos in the ecotypes used in this experiment. These results are in agreement with those of Fereol et al. [31] who showed that lower concentrations of 2,4-D (0.3–0.5 mg L^{-1}) resulted in a higher percentage of embryogenesis than higher concentrations of 2,4-D (1 or 1.5 mg L^{-1}), even in the presence of Kin. Casein hydrolysate and proline, which were present in all treatments, also positively influenced highly successful callus development, as noted by Khaleda and Al-Forkan [43]. Somatic embryos formed in clusters indirectly on the callus, in the form of pro-embryogenic masses (Figure 2a), or directly on parts of explants (Figure 2b).

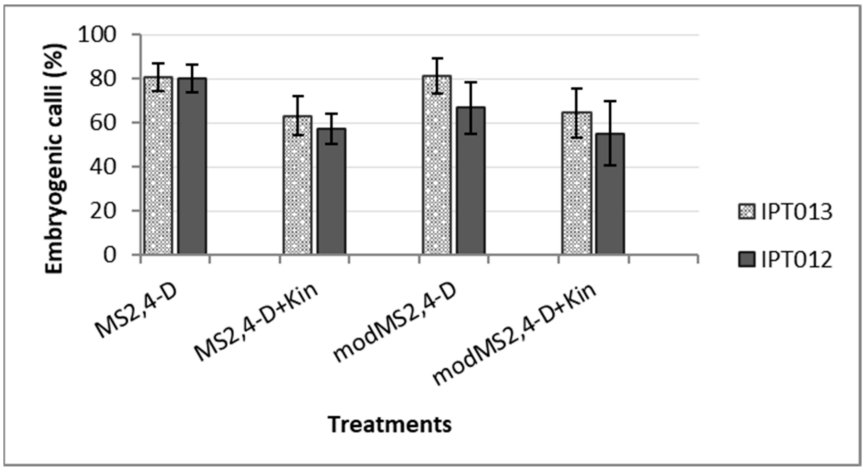

Figure 1. Percentage of embryogenic calli, as affected by medium composition (treatment) and ecotype (IPT013 and IPT012).

3.2. Plant Regeneration

Ten days after the first subculturing and light exposure of the explants, somatic embryos cultured on MS2,4-D or modMS2,4-D treatment started to germinate, mostly without radicle development (Figure 2c,d), and sometimes with coleoptile and radicle development (Figure 2c,e). Analysis of variance showed that both treatment and ecotype had a significant effect on regeneration efficiency (at $p < 0.05$) (Table 2), while their interaction was not significant. Plant regeneration efficiency, i.e., conversion of somatic embryos into plants, was the highest for somatic embryos originated from media containing a low concentration of 2,4-D as the only plant growth regulator (PGR), regardless of whether it was a full or modified MS medium (Table 2). Moreover, on media containing 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin, plant conversion did not begin until the calli were transferred to the MS2,4-D treatment. Regeneration then began, but with lower efficiency than in explants continuously cultured on MS2,4-D containing 0.1 mg L^{-1} 2,4-D.

Table 2. Number of plantlets regenerated per embryogenic callus, as affected by treatment and ecotype.

	No. of Plants/Embryogenic Callus ± SE		
Treatment	**IPT013**	**IPTO12**	***Mean***
MS2,4-D	3.8 ± 0.9	3.0 ± 1.0	3.4 [a]
modMS2,4-D	5.3 ± 1.6	2.3 ± 1.0	3.8 [a]
MS2,4 D+Kin→MS2,4-D	0.4 ± 0.3	0.2 ± 0.1	0.3 [c]
modMS2,4 D+Kin→MS2,4-D	2.6 ± 1.2	0.4 ± 0.2	1.5 [b]
Mean	3.0 [a]	1.5 [b]	

Values followed by the same letter are not significantly different according to Bonferroni tests at $p < 0.05$; SE-standard error. Regeneration was analysed and plantlets were repeatedly taken (depending on treatment) from day 35 of the experiment for the next four months. Values represent the average cumulative number of plants regenerated per embryogenic callus.

This is consistent with the known fact that high concentrations of 2,4-D impair embryo development and their subsequent conversion into plants [44,45]. Nissen and Minocha [46] suggested that the 2,4-D accumulation that occurs inside the globular embryos interferes with their ability to establish the internal auxin gradient that enables cell polarization. Consequently, 2,4-D (but also other auxins) disrupts the normal endogenous auxin balance and polar auxin transport that is important for bilateral symmetry during early plant embryogenesis [47,48]. It is possible that even such a low concentration of 2,4-D, as in the MS2,4-D treatment (0.1 mg L^{-1}) to which the somatic embryos were continuously exposed in this study, generally prevented bipolar germination of embryos (Figure 2d), which appeared only sporadically on the calli. Thus, this work confirmed the main prob-

lem of somatic embryogenesis, namely the development of a large number of abnormal embryos that cannot develop into a normal plant [49]. However, the possibility that some regenerated plantlets were formed by organogenesis cannot be completely excluded.

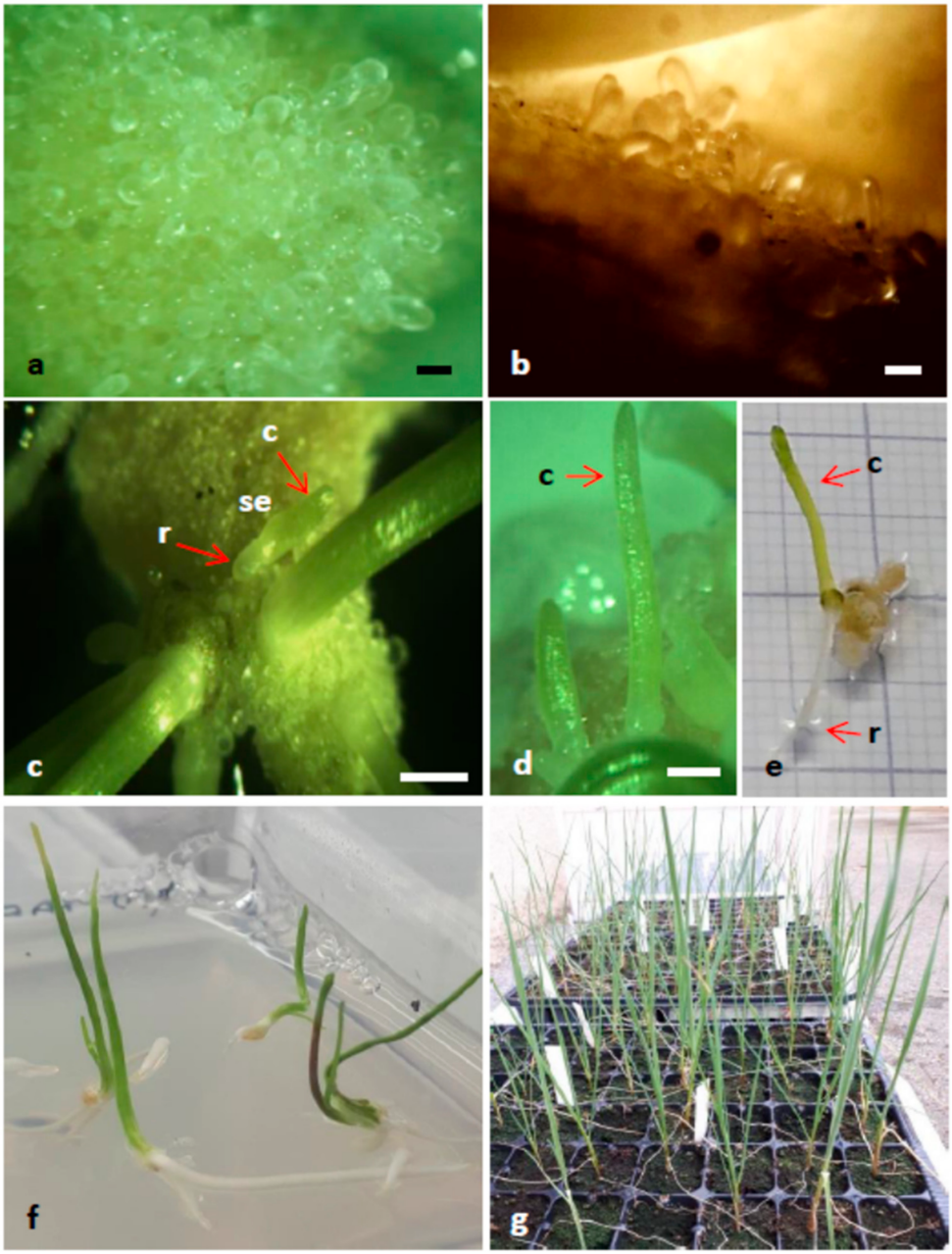

Figure 2. Pro-embryogenic mass with globular somatic embryos formed on the callus (**a**); globular somatic embryos formed directly on the explant (**b**); somatic embryos at different stages of development, from globular to germinated (**c**); somatic embryo with elongated coleoptile, without elongation of the radicle (**d**); germination of somatic embryo with simultaneous elongation of coleoptile and radicle (**e**); plantlets in the rooting medium (**f**); acclimatised plants (**g**). se—somatic embryo; c—coleoptile; r—radicle. (Scale bars = 1 mm in (**a**,**b**,**d**) and 5 mm in (**c**)).

Germinated embryos, with only coleoptiles developing, required the transfer of small regenerated plants to the rooting medium (Figure 2f). The developed plantlets could be separated from the callus very easily, without cutting. The regeneration potential of the somatic embryos was maintained for four months. After this period, the regeneration of the plants was very slow or stopped completely.

3.3. Rooting and Acclimatisation

All regenerated plantlets (germinated somatic embryos), with or without formed radicles, were subcultured on the rooting medium. The transfer of the regenerated plantlets lasted for four months and was carried out periodically from the 40th day after the start of the experiment. Rooting of the plants was performed on only one medium composition (MS supplemented with 0.05 mg L^{-1} NAA + 0.005 mg L^{-1} 2iP), but the success of rooting was analysed in relation to the previous treatment from which the plants originated (on which they were regenerated), as it could affect rooting efficiency. Moreover, no difference in pre-treatments was made between the full and modified medium, but only in relation to hormone content (MS2,4-D or MS2,4-D + Kin→2,4-D) (Table 3). Thus, plants of the same ecotype, bulb, and treatment (full or modified) were transplanted into the same Magenta vessels in the rooting medium. Analysis of variance showed that there were significant differences ($p < 0.05$) in rooting in relation to treatment (Table 3), while the influence of ecotype and ecotype x treatment interaction were not significant.

Table 3. Rooting and acclimatisation efficiency, as affected by previous medium/treatment.

Previous Treatment	Rooted Plantlets ± SE (%)	Acclimatised Plants (%)	Acclimatised Plants (N)
MS2,4-D	59.4 ± 3.3 [b]	64.0	122
MS2,4-D + Kin→MS2,4-D	89.0 ± 7.3 [a]	66.0	19

Values followed by different letter are significantly different at $p < 0.05$; SE-standard error.

In contrast to regeneration, which was significantly more successful on MS2,4-D treatment, MS2,4-D + Kin→MS2,4-D treatment as a previous medium showed a better effect on plant rooting (Table 3). Although Kin was present only during the formation of embryogenic calluses (in the medium preceding regeneration), the positive effect of Kin on rooting persisted. The positive effect of Kin on rooting was also reported by Sata et al. [32] when maximum rooting of garlic regenerated by somatic embryogenesis was obtained just on the medium supplemented with Kin (0.5 mg L^{-1}) alone. The problem, however, is that a relatively small number of plants regenerated on this treatment. The plants developed roots between the second and sixth week after transfer to the rooting medium. It can be assumed that these differences in time required for rooting are due to differences in the quality of the regenerated plants, i.e., the presence or absence of a radicle meristem. Plant acclimatisation was equally successful regardless of the treatment in which the plants regenerated, reaching up to 66% (Table 3). Khan et al. [30] in their study found that about 75% of the plants survived after transplanting from in vitro conditions to the substrate.

3.4. Virus Detection and Efficiency of Virus Elimination

The health status and presence of different viruses in the mother plants as well as in their regenerants determined by ELISA are shown in Table 4. A detailed summary of ELISA results for each mother plant and all regenerants included in the study is provided in Supplementary Table S1. In a previous study, a high percentage of clove infection with GCLV, SLV, LYSV and OYDV was found in both ecotypes used in this study [7]. However, we could not detect SLV in the mother plants used in this experiment; therefore, this virus was excluded from further tracking.

Table 4. The success rate of virus elimination by MS2,4-D and MS2,4-D + Kin→MS2,4-D treatments. The presence of viruses of the mother plants and their regenerants was determined by ELISA.

Mother Plant	Viruses Confirmed in Mother Plants			Virus Elimination Success Rate Determined in Regenerants (No. of Virus Free Plants/No. of Tested Plants)		
	OYDV	GCLV	LYSV	OYDV	GCLV	LYSV
				MS2,4-D treatment		
IPT013-2 *	+	+	+	1/11	2/11	2/11
IPT013-3	+	-	+	0/7	n/a	2/7
IPT013-6	+	-	+	4/16	n/a	7/16
IPT013-7	+	-	+	3/9	n/a	3/9
IPT013-8	+	-	+	3/13	n/a	3/13
Ecotype IPT013 virus elimination rate				11/56 19.6%	2/11 18.2%	17/56 30.4%
IPTO12-4	+	+	+	0/2	0/2	0/2
IPTO12-6	+	+	+	1/7	1/7	1/7
IPTO12-8	+	+	+	1/6	2/6	2/6
Ecotype IPT012 virus elimination rate				2/15 13.3%	3/15 20%	3/15 20%
Total virus elimination rate for MS2,4-D treatment				13/71 18.3%	5/26 19.2%	20/71 28.2%
				MS2,4-D + Kin→MS2,4-D treatment		
IPT013-2	+	+	+	4/6	3/6	5/6
IPT013-6	+	-	+	0/2	n/a	0/2
Ecotype IPT013 virus elimination rate				4/8 50%	3/6 50%	5/8 62.5%
IPTO12-8	+	+	+	0/3	0/3	0/3
Ecotype IPT012 virus elimination rate				0/3 0%	0/3 0%	0/3 0%
Total virus elimination rate for MS2,4-D + Kin→MS2,4-D treatment				4/11 36.4%	3/9 33.3%	5/11 45.5%
Total virus elimination rate for both treatments				17/82 20.7%	8/35 22.9%	25/82 30.5%

* The last number in the label represents the bulb. The experiment was started with explants of eight different bulbs of each ecotype.

According to ELISA test results, somatic embryogenesis was successful in eliminating OYDV, GCLV and LYSV in 20.7%, 22.9% and 30.5% of overall regenerated plants, respectively. Out of 82 ELISA-tested regenerants obtained from different mother plants with different virus combinations, 16 plants (19.5%) were free from all tested viruses.

Although confirmed in a limited number of plants, treatment with MS2,4-D + Kin→ MS2,4-D gave better results in virus elimination of OYDV, GCLV and LYSV compared to treatment with MS2,4-D. A possible explanation for this phenomenon is the presence of the cytokinin Kin in the medium for the induction of callogenesis and somatic embryogenesis. George and Sherrington [50] found that the meristematic nature of callus tissue can inhibit virus replication, especially in cytokinin-containing media. Recent research has shown that cytokinins activate the expression of the WUSCHEL (WUS) homeobox transcription factor [51] and Wu et al. [52] proved that WUSCHEL inhibits viral protein synthesis. In addition, more time (at least one month more) elapsed before plant regeneration began in embryogenic calli when treated with kinetin. Prolonged callus cultivation is known to reduce virus concentration in callus as demonstrated by Gambino et al. [21]. After four months of cultivation, Gambino et al. [21] detected at least one virus in embryogenic grapevine calli, whereas no viruses were detectable in callus tissue after eight months of cultivation. Nevertheless, grapevine plantlets obtained by somatic embryogenesis were virus-free regardless of the duration of callogenesis. In this study, however, only a portion

of the plants regenerated by somatic embryogenesis was virus-free, likely, depending on which part of the callus they were regenerated from. This is in agreement with the results of Wang and Hu [53] and Walkey [54] who found that calli originating from virus-infected plants are a mosaic of infected and uninfected cells. Since viruses could be translocated through plasmodesmata [55] and cytoplasmic connections between cells are known to occur in callus culture [56,57], translocation pathways through plasmodesmata could be a reason for virus-positive plantlets despite regeneration through somatic embryogenesis.

To confirm virus-free status and exclude possible false-negative ELISA results due to low virus titer and uneven distribution within the young plantlets, the virus-free status of regenerants was confirmed by RT-PCR (Figure 3). RT-PCR analysis confirmed the ELISA test results, i.e., the virus-free status of the analysed regenerants.

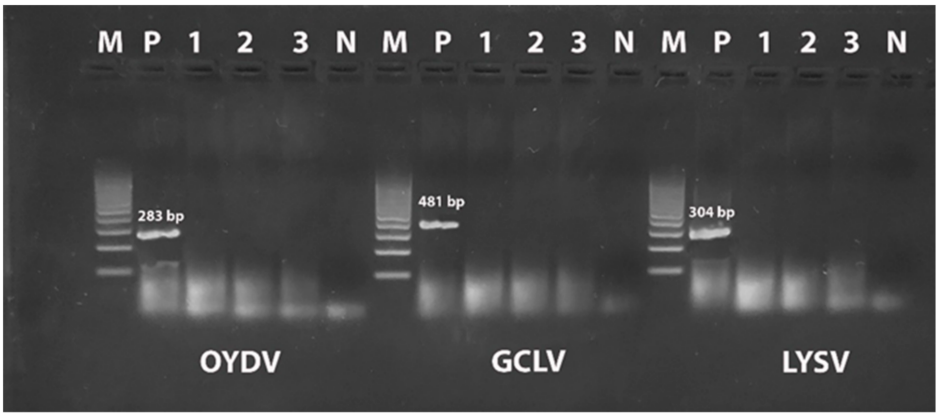

Figure 3. RT-PCR results performed on plants obtained by somatic embryogenesis. M—GelPilot 100 pb Plus Ladder (Qiagen), P—positive control (mother plants IPTO12-6, IPTO13-2 and IPTO13-8, respectively) with corresponding product size: OYDV—283 base pairs (bp), GCLV—481 bp, LYSV—304 bp; 1—virus-free regenerant IPTO12-6 MS2,4-D 3, obtained from the mother plant infected with all three viruses; 2—virus-free regenerant IPTO13-2 MS2,4-D + Kin 4, obtained from the mother plant infected with all three viruses, 3—virus-free regenerant IPTO13-8 MS2,4-D 5, obtained from the mother plant infected with OYDV and LYSV; N—negative control.

As previously mentioned, somatic embryogenesis has proven to be a promising and useful tool, especially in plant species with regeneration problems and problems with viral infections associated with vegetative propagation from infected mother plants. In this study, virus elimination success ranged from 13.3% to 62.5% depending on garlic ecotype, virus infection and embryogenesis treatment. This success of virus elimination by somatic embryogenesis is lower than the values obtained by Quainoo et al. [23] for cocoa trees or Damba et al. for cassava [24], but is comparable to the success rate of grapevine virus elimination as reported by Malenica et al. [22]. The obtained success rate is also comparable to the results of other methods of garlic virus elimination [11,13,58]. It is likely that a combination of clove thermotherapy and somatic embryogenesis or somatic embryogenesis on a ribavirin-containing medium would have a synergistic effect on a higher percentage of virus-free regenerated plants. To the best of our knowledge, this study describes for the first time the application of the somatic embryogenesis procedure for the production of virus-free garlic plants. These results could be useful in the production of healthy planting material of garlic. It could be particularly important for the protection of endangered garlic landraces around the world. There are many native garlic landraces and ecotypes that are neglected due to their deteriorated health status but are important for biodiversity conservation. We believe that virus elimination could contribute to their better field performance and thus to their popularisation and introduction into wider commercial production.

4. Conclusions

An efficient protocol for the regeneration of virus-free garlic plants through somatic embryogenesis was developed for two Croatian garlic ecotypes. There was no significant difference in the success of embryogenic callus induction in the four media compositions used. However, some limitations are worth noting. Conversion of somatic embryos on media containing 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin did not begin until calli were transferred to the medium supplemented with 0.1 mg L^{-1} 2,4-D alone. Nevertheless, conversion of somatic embryos to plants was significantly higher for the somatic embryos that originated from media containing 0.1 mg L^{-1} 2,4-D as the sole PGR compared to media supplemented with 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin. Plantlets regenerated on treatment MS2,4-D + Kin→MS2,4-D rooted with higher success compared to plantlets regenerated on the MS2,4-D treatment. The overall rate of virus elimination by somatic embryogenesis for both treatments and ecotypes was 20.7%, 22.9% and 30.5% for OYDV, GCLV and LYSV, respectively, as analysed by ELISA and confirmed by RT-PCR. This study, therefore, indicates that somatic embryogenesis could be a useful tool for the elimination of economically important viruses in garlic and suggests that this approach appears to be effective in the production of virus-free planting material.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050876/s1, Table S1: ELISA results performed on mother plants and the plants produced from them by somatic embryogenesis.

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