Suspension depletion approach for exemption of infected Solanum jasminoides cells from pospiviroids

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Despite numerous studies, viroid elimination from infected plants remains a very challenging task. This study introduces for the first time a novel ‘suspension depletion’ approach for exemption of Solanum jasminoides plants from viroids. The proposed method implies initial establishment of suspension cultures of the infected plant cells. The suspended cells were then physically treated (mild thermotherapy, 33 °C), which presumably delayed the replication of the viroid. The viroid concentration in the treated biomass was monitored weekly using pospiviroid-specific PCR. After 10–12 weeks of continuous treatment, a sufficient decrease in viroid concentration was observed such that the infection became undetectable by PCR. The treated single cells then gave rise to microcolonies on a solid culture medium and the obtained viroid-negative clones were further promoted to regenerate into viroid-free plants. Three years of accumulated experimental data suggests feasibility, broad applicability, and good efficacy of the proposed approach.

Keywords: Citrus exocortis viroid, plantlet regeneration, pospiviroids, suspension culture, thermotherapy, viroids

Introduction

Soon after the discovery of viroids by Diener (1971), these smallest known subviral pathogens received great attention worldwide. This was due to some of them causing appreciable economic damage to agriculturally valuable crops. Solanaceous plants, such as potatoes (Solanum tuberosum) and tomatoes (S. lycopersicum), are highly susceptible natural hosts of many viroids and display no form of natural resistance. Among them, the popular ornamental S. jasminoides is one of the most vulnerable to viroid infections and can host up to seven pospiviroid species simultaneously, including quarantined Potato spindle tuber viroid (PSTVd) and Citrus exocortis viroid (CEVd) (Owens & Verhoeven, 2009). Recent studies by Verhoeven et al. (2008) and Gottsberger & Suárez-Mahecha (2010) indicate high risk of symptomless infection of ornamental solanaceous plants by CEVd as a source of infection for crop plants, and the urgency of taking measures to control disease spread.

Pospiviroids are built solely of short strands of circular, single-stranded RNA (246–401 nucleotides) without any protein coats, which makes them intrinsically resistant to any kind of protein-targeting therapy (Diener, 1999; Ding, 2009). The spectrum of the remaining possible viroid-aimed treatments is further limited because of the plant cells’ own pool of small regulatory RNA molecules, which are hardly distinguishable from the parasitic viroid RNAs.

The secondary structure of most pospiviroids, including PSTVd and CEVd, under physiological conditions may be described as having a rod-like shape composed of unbranched series of short helices and small internal loops stabilized by hydrogen bonds (Riesner & Gross, 1985; Riesner & Steger, 1990; Flores et al., 2012). A pospiviroid RNA typically consists of an LH terminal domain, a pathogenic domain, conserved central domain, variable domain, and an RH terminal domain (Watson & Wang, 2012), all having thermodynamically stipulated rod-like secondary and additionally folded tertiary organization, resulting in their final equilibrium shape.

Replication of pospiviroids occurs in an asymmetric fashion via host cell proteins that also mediate invasion and spreading of pospiviroids. The viroid–host interactions are strongly dependent on the RNA sequence/structural motifs. Furthermore, for successful replication, pospiviroid RNAs have to adopt transient metastable conformations containing elements of local higher-order structure (Flores et al., 2012). Prominent among them are conserved hairpins, which mediate replication and movement of pospiviroids and are functionally involved in the so-called ‘kissing loop’ interaction between the palindromic tetraloops of two consecutive hairpin I motifs, with their stems forming a subsequently longer internstrand duplex (Gas et al., 2007; Flores et al., 2009).

Because replication of the viroids largely depends on their thermodynamically stabilized secondary and tertiary structure, it might be possible to delay and even arrest the replication of the viroid through induced (physical or chemical) disturbances in those structures. The plausibility of this assumption is justified by computer simulations of thermodynamically favourable secondary

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structure as a function of temperature, made by this working group (Fig. 1).

As shown in Figure 1, temperature-induced disturbances of the viroid RNA hydrogen bond network result in significant structural changes that, in turn, may dramatically affect the viroid replication process. With this premise in mind, various studies have been conducted in an attempt to eliminate viroids from host plants by means of either high or low temperature treatment of plant tissues, organs or whole plants (Hollings & Stone, 1970; Kovalskaya & Hammond, 2014). The relevance of temperature for viroid infections was experimentally demonstrated by numerous working groups who found that cold- and heat-therapy can have opposite effects on viroid–plant systems (El-Dougoud et al., 2010; Kovalskaya & Hammond, 2014). There have been varying degrees of success in treating viroid-infected plants by means of thermotherapy (Barba et al., 2003). It has been reported that the concentration of CSVd was reduced in chrysanthemum grown for 2 months at low temperatures (10 or 20 °C) but increased to that of control (untreated) plants within weeks once it was moved to a normal glasshouse temperature (Chung et al., 2006).

In another study, the persistent heat treatment of the chrysanthemum plant at 37 °C elicited damage to leaves or shoots of in vitro explants resulting in a decreased survival rate with an increase in the duration of heat treatment (Jeon et al., 2012). The combination of thermotherapy with meristem-tip culture brought more promising results, as well as a better chance of viroid reduction in infected plants (Hollings & Stone, 1970; Lizarraga et al., 1980; Kovalskaya & Hammond, 2014). However, the efficacy of most of the proposed approaches, including chemotherapy, is greatly reduced due to the persistence and transfer of remaining viroids within a living plant. Therefore no treatment process has become generally accepted and broadly recognized as capable of eliminating pospiviroids from infected plants in an efficient and reliable way.

This study has developed and tested a novel approach aimed at elimination of pospiviroids from infected S. jasminoides plants. The proposed method features application of thermotherapy not on the infected whole plants, but on the suspension culture of infected plant cells. The key idea of the approach can be described in five basic steps:

(i) Plant cells in the suspension culture initially have a different number of viroids per cell and cannot effectively share viroids. This is because the traffic of viroids from an initially infected host cell into neighbouring cells requires intercellular junctions (Ding et al., 1997).

(ii) As cells proliferate under conditions that suppress viroid replication, the number of viroids per cell decreases (approximately by 50%) with each generation.

(iii) Given enough time, a certain non-zero fraction of viroid-free cells will appear in the suspension and will gradually increase in number, due to suppressed viroid replication.

(iv) After successful depletion of the cell population with respect to viroid copies, monitored and confirmed by regular PCR tests, each viable cell, being separately placed on top of an agar culture medium, can give rise to a microcolony of its clones.

(v) Isolated microcolonies originating from viroid-free cells will retain this state and, after appropriate PCR examination, can be used for regeneration of new viroid-free plants.
A more detailed description of the listed steps is provided below with their graphical representation given in Figure 2.

Materials and methods

Plants

Solana jasminoides, also called the potato vine, originates from South America and is considered to be extremely robust and easy to maintain at different temperatures. Starting from September 2013 until March 2017, around 20 plantlets of S. jasminoides were received every 6 months from Horticulture Hanka, Kempen, Germany. When received, the plantlets did not show any symptoms of disease and, after routine tests for viroids, were employed for callus induction. These donor plants were cultivated ex vitro under room conditions (22–25 °C). In total 143 plantlets of cultivars Lillirom (48 plantlets), Den Haag (33), London (37) and Bluerom (25) were included in the study.

Callus induction

For the initiation of callus culture, explants, preferably from pre-existent buds/meristems such as shoot tips and nodal segments (c. 1 cm), were taken from donor plants using sterile scalpels. Prior to introduction into the culture medium for callus induction, the explants were sterilized by submerging them into water supplemented with 0.5% Tween 20 for 1 min, followed by a double rinsing with autoclaved tap water, a subsequent submerging into 3% NaOCl for 1 min, and finally followed by a triple rinsing with autoclaved water.

The prepared explants were placed onto an agar-solidified Murashige and Skoog (MS) medium (Duchefa Biochemie B.V.) pH 5.6–5.8, supplemented with 12.4 g L⁻¹ sucrose, 1.5 g L⁻¹ polyvinylpyrrolidone PVP-40 (Carl Roth GmbH) and 2.5 p.p.m. 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma Aldrich Co.) and 5.8, supplemented with 12.4 g L⁻¹ sucrose, 1.5 g L⁻¹ polyvinylpyrrolidone PVP-40 (Carl Roth GmbH) and 2.5 p.p.m. 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma Aldrich Co.) and incubated at an ambient temperature of 22 °C and 95% relative humidity for 4–6 weeks in darkness. The callus was then removed from the explant and subcultured onto a fresh agarized medium for an additional 4 weeks in darkness at ambient temperature.

Establishment, maintenance and temperature treatment of cell suspension cultures

Each cell suspension culture was established by transferring one or two 35-day-old calli (1.0–1.3 g biomass) into 125 mL polycarbonate Erlenmeyer flasks with baffled base filled with 20–25 mL of liquid MS media supplemented with 1.5 mg L⁻¹ 2,4-D. The flasks were equipped with a vented Duocap system (TriForest Labware Co.). In a single experimental run, 18–20 suspensions were established for each cultivar. After all the suspensions were tested to be pospiviroid-positive, eight of them were labelled as the control group and were thereafter cultured under optimal conditions of temperature (22–25 °C). The other eight suspensions constituted the treatment group, which was continuously subjected to the mild thermotherapy (33 °C). The agitation rate (90–130 rpm) and light exposure (in darkness) were kept the same for both groups.

For all groups, subculturing was done by the transfer of 5 mL of cell suspension into 20 mL of fresh medium using sterile, wide-bored 5 mL pipettes. During the routine medium exchange, every 2 weeks, the suspension cultures were refined by filtration through a sterile gauze bandage. This procedure was aimed at enriching the suspension culture with single cells. Every 2 weeks, the culture medium osmolality was examined using the Osmomat 030-D (Gonotech Co.) and maintained within 210–240 mOsm (where 1 Osm = 1 mole of solute that contributes to the osmotic pressure of a solution).

The entire treatment series (11–12 weeks) was repeated at least three times for each cultivar, each time beginning from freshly prepared callus.

Cell counting and viability tests

In order to evaluate the culture proliferation rate, direct cell counting using a haemacytometer (Neubauer chamber) was conducted. In brief, 40 U (10 μL) of pectinase solution from Aspergillus aculeatus (Sigma Aldrich) was added to 1 mL of each cell suspension sample. The suspension cultures were then incubated at 25 °C under constant agitation of a 300 rpm shaker overnight. Afterwards, the samples were centrifuged at 280 g for 5 min and the supernatant was removed. Subsequently, 250 μL of fresh suspension medium was added to the pellet and the cell

Figure 2. Schematic illustration of the principal steps comprising the proposed suspension depletion approach. From left to right: taking an explant from the infected plant; callus formation on solidified MS agar medium; establishment of suspension cultures that undergo treatment, with efficacy monitored by PCR; after sufficient decrease of the viroid concentration in the suspension, microcolonies are formed on an agarized medium; from PCR-negative clones, new viroid-free plants are regenerated. In all steps, viroid content is routinely tested by PCR. [Colour figure can be viewed at wileyonlinelibrary.com]
Viroid identification and detection by RT-PCR

For pospiviroid RNA detection and quantitation, RT-PCR was performed using the kit from QIAGEN and primers provided by Life Technology GmbH/Thermo Fischer Co. In parallel, specific PSTVd detection in the samples was conducted according to Boonham et al. (2004). Primer sequences are shown in Table 1.

RNA extracts (2.5 μL) were incubated at 65 °C for 8 min and chilled on ice for 3 min, after which 7.5 μL of RT mixture was added to provide a final concentration of 20 ng μL−1 of the reverse primer (Table 1), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM diithiothreitol (DTT), 2.5 mM MgCl2, 1 μM of each dNTP, 5 U of RNasin ribonuclease inhibitor (Promega Corp.), and 100 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega Corp.). Samples were incubated at 50 °C for 30 min and 95 °C for 2 min. The resulting cDNA was used as a template for PCR that was performed on an iCycler (Bio-Rad) as described by Verhoeven et al. (2008).

Once the process was completed, the samples were cooled down and stored at −80 °C for later analysis. The amplified products (8 μL) were electrophoresed on a 1.5% agarose gel containing SYBR Safe (Invitrogen Co.) diluted 1:10 000 in TBE buffer. Visualization and analysis of the bands was done using the Gel-Imager (Bio-Rad). The molecular weight of the respective PCR products was determined with the aid of a GeneRuler 1 kb DNA Ladder and 100 bp DNA Ladder (Thermo Fisher Scientific GmbH).

The viroid type was identified by an ISO 17025-certified quality-assured testing laboratory in the Leibniz-Institut DSMZ GmbH in Braunschweig, Germany by sequencing of amplification products and comparison with sequences in genome databases (Data S1).

### Table 1 Primers used in this study.

| Primer | Sequence (5’–3’) | Position |
|--------|-----------------|----------|
| Pospi (A) RE | AGCCTCAGTTGTATCCACCAGGT | 261–283 |
| Pospi FW | GGGATCCCGCGGGAGAAC | 86–102 |
| PSTVd 157 FW | ACTCTGTCGCGGCTGGGCA | 114–135 |
| PSTVd 158 RE | AATTCCGCGCAGAACAGGGT | 128–149 |

Induction of microcolonies and plantlet regeneration

After sedimentation of the suspension cultures, most of the supernatant liquid was removed to achieve a higher concentration of cells. Aliquots were routinely monitored on a suitable microbiological medium indicating potential bacterial or fungal infections. Two millilitres of the resuspended callus cultures were transferred to sterile 55 × 15 mm polystyrene Petri dishes (Carl Roth GmbH) and mixed with the same amount of liquid MS media supplemented with 6-benzylaminopurine (BAP) and 9-naphthalene acetic acid (NAA), 1 and 1.5 p.p.m., respectively, or 2,4-D and NAA (all reagents from Duchefa Biochemie B.V.), 2 p.p.m. of each. These subcultures were incubated at 24 °C in the dark with a complete exchange of media every 7–10 days.

After 5 weeks of subcultivation, the microcalli were transferred to a modified solid half-strength MS medium supplemented with BAP and 2,4-D, 1 and 0.1 p.p.m., respectively. Incubation was carried out for 3 weeks at 25 °C in the dark; meanwhile the callus cells were transferred to the same fresh medium after 2 weeks.

Finally the callus cultures were transferred to a modified solid MS medium supplemented with zeatin, kinetin and indole-3-acetic acid (all from Duchefa Biochemie B.V.), 1, 0.5 and 0.15 p.p.m., respectively. Again, passages of the callus cultures onto the same fresh medium were carried out every 2 weeks; the callus cultures were then incubated, at 25 °C and 2000 lux light exposure following a 16 h long day, until plantlet regeneration. Under the same conditions, the regenerated plantlets were separated from the callus and propagated *in vitro*.

For better rooting of the regenerated *in vitro* plantlets, they were alternatively put onto solid quarter-strength MS medium supplemented with 0.1 p.p.m. indole-3-butyric acid and half-strength MS medium supplemented with IBA plus NAA, 4 and 0.1 p.p.m., respectively. After 3 days of incubation in the dark at 25 °C, the plantlets were cultivated at the same temperature under dim light following a 16 h long day until roots became visible.

The rooted regenerated plantlets were pricked out into Preform plant plugs (Jiffy Products International), cultivated under a foil tent in the greenhouse, and acclimatized to *ex vitro* conditions through stepwise ventilation.

Statistics

For statistical analysis, the samples were made at least in triplicate and all experiments were repeated multiple times. Respective table values show the standard error of the mean (SEM).

Results

The practical steps of the proposed treatment scheme are illustrated in Figure 3. The explants obtained from an infected *S. jasminoides* plant (Fig. 3a) were first used for induction of the callus culture on an agarized medium.
(Fig. 3b), which in turn gave rise to the suspension culture (Fig. 3c). The treatment groups, consisting of eight flasks for each cultivar, were subjected to mild thermography in a thermostat for 10–12 weeks (Fig. 3d). After the confirmation of the viroid elimination, viroid-free plantlets were regenerated (Fig. 3e) from the suspended cells.

Together with the obvious requirement of avoiding contamination problems (which is notoriously escalated in the case of culturing suspended cells), the suspension depletion approach relied primarily on permanent and appropriately quick cell proliferation in suspension (Fig. 3c,d) and secondarily on regeneration of plants from previously suspended cells. The latter was successfully achieved by careful adjustment of the solidified culture medium composition.

Osmolality, cell count and viability upon thermotherapy treatment

In previous pilot studies (authors’ unpublished data), 33 °C was found to be the most suitable temperature both in terms of *S. jasminoides* proliferation and for reduction of viroid concentration. Higher temperatures of incubation resulted in significant suppression of cell proliferation. As the cells were cultivated under stressed conditions upon treatment, their vitality and growth rate needed to be carefully addressed and regularly checked. In this study, micro-litre amounts of the suspension medium were extracted every 2 weeks to monitor treatment-induced changes in osmolality, cell concentration and viability. Typical measured values exemplified by a single Lilliroi treatment group (eight suspensions) are presented in Table 2.

In all groups, the osmolality, being dependent on numerous factors, remained fairly constant (210–240 mOsm) through the whole course of the treatment. On the one hand, as the cells proliferated and culture medium components were metabolized, the osmolality tended to decrease. However, on the other hand, this effect was counteracted by partial loss of water due to evaporation. An appreciable fluctuation of the data was introduced due to the 2 week cell passaging cycle. Cell counting was especially influenced by suspension filtration through a sterile gauze bandage during the routine medium exchange.

Heat stress conditions resulted in an increased number of dead cells (as exemplified in Table 2) and correspondingly enhanced the degradation level of the extracted RNA in the treated groups, for all tested cultivars. However, the overall results appeared satisfactory because the measured $A_{260}:A_{280}$ ratio of $c.2.0$ is generally accepted as pure (Tzertzinis et al., 2001).

The concentration of cell biomass in the treatment groups reached 170–195 mg mL$^{-1}$ prior to the start of the plantlet regeneration stage. In comparison to the biomass concentration directly after the establishment of the suspension culture (48–53 mg mL$^{-1}$), these values suggest that sustainable cell growth was steadily observed at the treatment temperature (33 °C).

Viroid RNA detection, identification and quantification

The efficacy of the applied treatment method in respect to exemption of infected *S. jasminoides* cells from pospiviroids was evaluated using pospiviroid-specific RT-PCR. After 10–12 weeks of continuous treatment of

![Figure 3](https://example.com/f3.png)

**Figure 3** (a) *Solanum jasminoides*; (b) callus induced from explant on solidified MS agar medium; (c) suspension cultures of *S. jasminoides*; (d) subculturing of suspension cultures under thermal treatment; (e) a plantlet regenerated from suspension culture. [Colour figure can be viewed at wileyonlinelibrary.com]
suspended samples, a dramatic reduction of pospiviroid concentration was observed in all cases (Fig. 4). The generic pospiviroid RT-PCR yielded an amplification product of the expected size for all untreated callus and suspension (control) samples, whereas the cell suspensions that had been incubated at 33 °C for 12 weeks displayed no viroid-specific bands. Among the PCR-tested samples, neither the plantlets taken in the study (143 plantlets), the control samples (approx. 85), nor the treated samples (approx. 80) displayed PSTVd-specific amplification.

After the whole treatment procedure was successfully performed six times, each time beginning from freshly prepared infected callus, two control samples (Bluerom and Lillirom) and two corresponding treated samples were blindly analysed by an external ISO 17025-certified quality-assured test laboratory at Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) by using two standard PCR protocols (Data S1). According to the DSMZ report, pospiviroids were detected in both control samples, whereas both treated samples were found to be viroid-free. The sequencing done by GMAI/HZI Braunschweig, Germany, identified the infectious agent in the control samples as CEVd.

**Induction of microcolonies and plantlet regeneration**

A considerable proliferation of the callus cells of all cultivars took place during the subcultivation from the liquid media and onto a solid medium. These results were successfully achieved from a variety of calli, where plantlet regeneration typically occurred after 17–19 weeks of incubation. During this time, the callus was exposed to light on the zeatin-containing medium. In general, microcolonies tended to be regenerative when the solid callus appeared bright green in colour (Data S2).

Regenerated plantlets were separated from the callus and could be successfully established as stable clone lines by in vitro cultivation and propagation at 25 °C with 2000 lux light exposure and 16 h daylength.

Rooting of the regenerated in vitro plantlets was achieved with both media with rooting rates of 50% (quarter-strength MS with IBA) and 83.3% (half-strength MS with IBA and NAA). After 2 weeks of cultivation under dim light, the roots had a suitable length for transferring the plantlets from agar into soil. Finally, the plantlets were successfully adapted to ex vitro conditions and sprouted to complete plants in the greenhouse (Data S2).

**Discussion**

*Solanum jasminoides* is an ornamental plant and often infected with pospiviroids, which upon unintended viroid transfer onto agricultural crops, may result in significant economic loss. So far, effective infection control methods for viroid diseases are largely absent, so that an outbreak...
of viroid infection forces massive host plant eradication (Jeon et al., 2012; Sastry & Zitter, 2014). The aim of this study was to test and validate a novel suspension depletion approach for viroid elimination from plant cells. The proposed method is based on popular but perhaps not very efficient thermal therapy of the infected plants, bringing a new principle feature of cell treatment in the suspension culture conditions. In contrast to infected plant tissues where intercellular trafficking of viroid occurs via plasmodesmata, individual cells in a suspension culture are disjointedly distributed in the culture medium and have an autonomous cell cycle (Ding et al., 1997; Gstraunthaler & Lindl, 2013).

Suspension cell cultures of S. jasminoides were cultured at 33°C on the assumption that it could arrest the viroid replication through physically induced rearrangements of the RNA secondary/tertiary structure. At the same time, the chosen mild thermal treatment protocol apparently allowed suspended plant cells to remain viable and proliferate in a sustainable manner. Considering the absence of adjacent cells in the suspension cultures and the viroid replication presumably being temporarily arrested/delayed, the proportion of viroid-infected cells would decrease as the cells divide. Upon subculturing, the number of viroid-infected cells (and the total number of viroids) would also decrease due to removal of the surplus biomass.

Indeed, the results showed that with continuous culturing under elevated temperature, cell suspensions became depleted from viroids. After 12 weeks of treatment, the specific detection of pospiviroids was carried out by RT-PCR as described by Verhoeven et al. (2008). In all treated samples, a total elimination or at least a dramatic reduction of pospiviroid concentration was observed, whereas the viroid concentration in the control groups remained unaffected. During the study, eight successful viroid elimination runs were accomplished by means of the proposed suspension depletion method in the cultivars Lillirom, Den Haag and London, with experimental data externally proofed by the DSMZ. Based on the obtained results, collected over a 3-year period, this study claims successful application of the suspension depletion approach for CEVd elimination from the infected cells.

The main criterion that determined the thermotherapy duration (10–12 weeks) was the absence of detectable viroids in the treated samples. However, there were two other factors that influenced the span of the treatment and occasionally lead to premature treatment termination: (i) microbial contamination and (ii) gradual decrease in cell density. In the authors’ opinion, these might be the major problems faced upon practical implementation of the proposed treatment scheme.

Sucrose concentrations between 2% and 3% have been reported to be optimal for many suspension cultures (Saxena et al., 1981; Huda et al., 2007; Shirin et al., 2007). In the present studies, this culture medium composition resulted in a steady state osmolality of around 220 mOsm and provided a sustainable cell culture.

The regeneration of plantlets from suspension cultures is a very important step for the practical implementation of the proposed viroid elimination scheme. Different combinations and concentrations of phytohormones have been reported to lead to regeneration of plants from protoplasts, callus, somatic embryos and explants of solanaceous species including Solanum melongena, S. surattense, S. trilobatum and S. tuberosum (Saxena et al., 1981; Swamy et al., 2005; Dhavala et al., 2009; Swamynathan et al., 2010). To the authors’ knowledge, this is the first report on plant regeneration from S. jasminoides callus cells, and the influence of the phytohormone zeatin on doing this is consistent with literature data about S. tuberosum (Shepard & Totten, 1977; Bríza & Machová, 1991; JayaSree et al., 2001; Rahman et al., 2006) and S. melongena (Rahman et al., 2006). Achieved regeneration of plants from suspended cells suggests practical applicability of the method. However, time seems to be a critical factor in the whole plant regeneration process because the procedure extended over almost 5 months. Ongoing experiments with different media and hormone compositions might lead to an acceleration of this important step.

This article reported the overall concept as well as the main results on exemption of S. jasminoides from CEVd viroid using the suspension depletion approach. In summary, this work demonstrated the feasibility of (i) the proposed suspension depletion approach, and (ii) plantlet regeneration from suspension cultures. Successful plantlet regeneration from both treated and untreated cultures of cultivars Lillirom, Den Haag and London has been achieved so far; further improvement of cell culture protocols are in progress.

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