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LATENT VIRUSES ELIMINATION OF *MALUS DOMESTICA* BORKH TREE ROOTSTOCK IN VITRO

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SUMMARY

The plants' recovery from the clonal rootstock of apple tree 62-396 from the Apple (*Malus domestica* BORKH) chlorotic leaf spot virus (ACLSV) and Apple stem grooving virus (ASGV) succeeded in using antiviral drugs (AVD), ribavirin (standard), salicylic, and jasmonic acids, at air temperatures of +20 °C and +38 °C. Magnetic pulse processing (MPP) application used a device with a frequency of 200 Hz. With the temperature of +20 °C, the possibility of using AVDs for 90 days was definitive. The combination of temperature (+38 °C) and MPP (without AVD) and temperature (+38 °C) and salicylic acid (81.3%) provided the highest survival rate. The increased temperature (+38 °C) also inhibited shoot proliferation, leading to a decrease in shoot number by 2.9 times in the control and by 1.1–1.5 times in the variants with AVD. MPP reduced the inhibitory effects of high temperature in the control. The explants' yield of the clonal rootstock of apple tree 62-396 was free from latent viruses in vitro using various methods of rehabilitation, which was 50%–100%. A low virus infection index (1.2) was notable during complex therapy with the use of AVD (salicylic acid + jasmonic acid), thermotherapy, and MPP.

Keywords: Apple (*M. domestica* BORKH) viruses' elimination, in vitro thermotherapy, in vitro chemotherapy, in vitro magnetic pulse processing, apple clonal rootstock

Key findings: In breeding programs and planting mother trees, it is crucial to use the clonal apple (*M. domestica* BORKH) rootstocks that have already received treatments against viruses using in vitro chemotherapy (salicylic acid 30 mg/l + jasmonic acid 2.5 mg/l), thermotherapy (+38 °C), and magnetic pulse processing (200 Hz).

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INTRODUCTION

Apple (*Malus domestica* BORKH) tree cultivars used in breeding programs and for planting mother trees with subsequent propagation must be free from major harmful viruses (Keshavarz and Hajnajari, 2021). The Russian Federation established the categories of planting material. The need to produce planting material for horticultural crops should be free of Apple chlorotic leaf spot virus (ACLSV), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), and Apple mosaic virus (ApMV), and other harmful organisms. In the Russian Federation, the following categories of apple tree planting material produced were initial plants, basic plants, and tested material.

For increasing high-quality apple production, certified planting material is a challenging issue in commercial production requiring expansion of the growing area under highly productive plantings. In the Russian Federation, the production of superior quality planting material mostly comes from breeding and seed centers. The establishment of breeding and seed production centers emerged at major research and educational institutions, equipped with modern diagnostic equipment for the identification and management of viruses and other harmful pests. As and when required, the use of techniques to improve plant health is also applicable to rid plants of viruses. Healthy planting material undergoes rapid propagation in compliance with all necessary planting certification requirements according to international standards (Plants Certification Scheme, 2021) and Russian standards (GOST R 59653-2021).

When obtaining apple tree planting materials, the rootstock type is highly valuable. Usually, using semi-dwarf and dwarf clonal rootstocks serves for the formation of compact crowns. One of the primary dwarf-apple rootstocks for the Central and Northern zones of the Russian Federation is the 62-396 rootstock. It has characteristics of medium-strength wood, a high frost and cold-resistant root system (-15 to -16 °C), and better propagation potential, with early fruiting in grafted trees (Trunov *et al*., 2016).

With a large volume of propagation from untested planting materials, clonal rootstocks mostly emerge with the highest levels of virus contamination. The major latent viruses prevalent on clonal apple rootstocks ranged from 24% to 39.1% (Upadyshev *et al*., 2014, 2017, 2021a, and b). On the dwarf apple rootstock 62-396, the incidence of ACLSV reached 69.7% under Russian conditions (Upadyshev, 2022). In the planting collection of Iran, 71.4% of apple tree samples showed infections with the ACLSV virus, 47.5% with ASPV, and 18.5% with ASGV (Keshavarz and Hajnajari, 2021). Meanwhile, for Korea, 47.6% of apple trees exhibited infection of three different viruses, i.e., ACLSV, ASGV, and ASPV, while not detecting ApMV (Cho *et al*., 2016). In apple trees, significant damages resulted in ACLSV, ASGV, ASPV, and ApMV (Prikhodko and Magomedov, 2011). These specified viruses must not be present in planting materials of the certified category according to the Russian Standard (GOST R 59653-2021).

The primary method of virus elimination is dry-air thermotherapy, carried out at a temperature of +38 °C (Plants Certification Scheme, 2021). However, dry-air thermotherapy ex situ often leads to the highest mortality rate of plants being treated, aside from being labor- and energy-intensive, and with some cultivars and viruses, it obtained a low yield from healthy plants (Prikhodko and Magomedov, 2011; Upadyshev *et al*., 2019; Li *et al*., 2020). Therefore, an alternative is the in vitro thermotherapy using compact thermal chambers (thermostats and climatostats) (Wang *et al*., 2018). However, thermotherapy is not effective enough against heat-stable viruses, ASPV and ASGV.

The in vitro chemotherapy advantages include the possibility of using explants larger than 1 mm, a considerable reduction in virus elimination time, and the highest fruit yield from virus-free plants (Prikhodko and Magomedov, 2011; Upadyshev *et al*., 2019). Using ribavirin was the most effective for chemotherapy. The mechanism of ribavirin's action is to inhibit the biosynthesis of viral RNA replication. Ribavirin at concentrations of 25–100 mg/L resulted in the production of ASGV-free apple regenerants both in integration with the in vitro thermotherapy and without thermotherapy (Cieslinska and Zavadska, 1999). The disadvantages of ribavirin include its phytotoxicity and the possibility of mutations. Salicylic acid and its precursor, jasmonic acid, also play an important role in the development of acquired specific resistance to various pathogens, including viruses (Ding and Ding, 2020; Zhu *et al*., 2024; Zhang *et al*., 2025). Increased expression of genes encoding salicylic and jasmonic acids enhanced the tobacco's resistance to Tobacco mosaic virus (TMV) (Zhu *et al*., 2014). Salicylic and jasmonic acids expressed low phytotoxicity, favoring environmental safety.

Magnetic pulse processing (MPP) using devices that generate magnetic induction pulses can be applicable to treat different plant viruses. MPP is one of the new methods of healing from viruses. The mechanism of action of magnetic treatment on viruses consists of increasing the synthesis of phenolic compounds and the development of non-specific plant resistance. Magnetic pulse processing eliminates growth inhibition and improves environmental safety. However, the effectiveness of magnetic therapy depends on the type of virus and the frequency of treatment, which, in some cases, can reach 80% (Donetskikh *et al*., 2017; Upadyshev *et al*., 2021a, b).

Based on the different types of viruses, the methods used to improve apple tree health could vary and require refinement predicated on the biological characteristics of the crop and properties of specific viruses. The consequent study aimed to determine the effectiveness of in vitro thermotherapy, chemotherapy, and magnetic pulse processing in the treatment of the clonal dwarf apple rootstock 62-396 against primary harmful viruses.

MATERIALS AND METHODS

Cultivation and antiviral therapy of in vitro explants

Microshoots measuring 5–10 mm of apple (*M. domestica* BORKH) underwent culturing on the MS medium supplemented with 6-benzylaminopurine (6-BAP) at the concentration of 1 mg/L (Murashige and Skoog, 1962). Sixteen explants planted in each treatment totaled 192 explants in the whole experiment. The antiviral drugs (AVD) used were ribavirin (standard) at a concentration of 40 mg/L, salicylic acid (30 mg/L), and jasmonic acid (2.5 mg/L). The control has no treatment. The effects on explant survival, growth parameters, and virus-free explant yield entailed studies with two air temperatures (+20 °C and +38 °C). Explants' exposure to heat (+38 °C) for three months used a KS-200 SPU climate control system (Russian Federation). In all experimental conditions, explants received culturing under 3000-lux illumination and a 16-hour photoperiod. Magnetic pulse processing of the explants proceeded using an AMIS-8 magnetic pulse stimulation device at a frequency of 200 Hz (Donetskikh *et al*., 2017) (Figure 1). After completing the therapy, the transfer of explants continued onto the MS medium without antiviral drugs, before the addition of 1 mg/l 6-BAP and culturing at a temperature of +20 °C, illumination of 3000 lux, and a photoperiod of 16 hours. After three months of cultivation, the explants underwent ELISA (enzyme-linked immunosorbent assay) testing for the presence of viruses.

Chemicals

The ELISA for Apple stem grooving virus (ASGV) and Apple chlorotic leaf spot virus (ACLSV) continued using diagnostic kits (immunoglobulins, conjugates, and positive and negative controls) from Loewe, Germany. All other chemical substances selected for the analysis were of analytical quality (minimal purity 99%) sort, bought from Sigma-Aldrich (USA) and Panreac Applichem (Spain).

Sample preparation

Microshoots served as samples for analysis. Microshoot samples incurred homogenization in a sample (conjugate) buffer—PBS buffer (pH 7.4) with the addition of 2% polyvinylpyrrolidone and 0.2% bovine serum albumin. The samples' homogenization had the

Figure 1. Magnetic pulse processing of apple clonal rootstock 62-396 explants using the AMIS-8 apparatus.

ratio of 200 mg of leaves per 3 ml of sample buffer. After homogenization, the leaf samples sustained centrifugation on a Beckmann J2-21 M/E centrifuge (UK) at 2500 rpm for 20 min at +4 °C. The resulting supernatant served for plating on Costar (USA) 96-well microplates in an amount of 200 µl per well in two replicates.

Enzyme-Linked Immunosorbent Assay (ELISA)

Testing of clonal dwarf apple-rootstock plants 62-396 for ACLSV and ASGV succeeded in accordance with the methodological directions (Upadyshev *et al*., 2013). Coating buffer (pH 9.6) for immunoglobulins contained 1.59 g l^{-1} Na_2CO_3, 2.93 g l^{-1} $NaHCO_3$, and 0.2 g l^{-1} NaN_3 of distilled water. The coating buffer, upon application to the microplates, entailed incubation in a refrigerator at +4 °C for 16 hours. Microplates received washing three times with PBS wash buffer (pH 7.4). After washing, the applied samples onto the microplates sustained incubation at +4 °C for 16 hours. After incubation, washing the microplates four times used the PBS wash buffer (pH 7.4). The conjugate dilutes 1:200 in the conjugate buffer. The conjugate, as applied to the microplates, reached further incubation in a TS-1/80 SPU thermostat (Russia) at +37 °C for four hours. Covering the microplates with lids preceded thermostating. Afterward, washing the microplates three times used PBS wash buffer (pH 7.4), which continued with the addition of the 4-nitrophenylphosphate to the substrate buffer (97 ml l^{-1} of diethanolamine, 0.2 g l^{-1} NaN_3 of distilled water, pH 9.8) ml^{-1}. The substrate buffer application to the microplates at +20 °C ensued in the dark. After two hours, recording the analysis results on a Stat Fax 2100 (USA) plate photometer had the wavelength of 405 nm (as the main wavelength) and 630 nm (as the auxiliary wavelength).

Data recorded and statistical analysis

The study determined the virus infection index of leaf samples. The infection index calculation was the ratio of sample extinction to the extinction of negative control, i.e., 1.0 to 1.59 (no virus), 1.6 to 1.99 (probable infection), and 2.0 and higher (reliable infection). All data entailed expression as mean values of the infestation index with standard deviation (SD), with the MS Excel (Microsoft Excel, v.2016) software used for statistical analysis.

RESULTS

By improving the plant health of clonal dwarf apple (*M. domestica* BORKH) rootstock 62-396 in vitro, the results revealed the survival rate of explants was reliant on the temperature, the type of antiviral drug, magnetic pulse processing, and the duration of cultivation (Table 1). With the cultivation temperature of +20 °C in nutrient media containing AVD for 45 days, the highest explant survival emerged,

Table 1. Survival rate of apple tree 62-396 explants' clonal rootstock in the viruses' elimination process, depending on the action of chemical and physical factors, and duration of cultivation on a nutrient medium (%).

Physical factors of cultivation	Antiviral drug							
	Ribavirin (standard)		Salicylic acid		Salicylic acid + jasmonic acid		Control (without drug)	
	45*	90	45	90	45	90	45	90
Temperature + 20 °C	81.3	68.8	87.5	68.8	93.8	62.5	100	75.0
Temperature + 38 ºC	56.3	18.8	81.3	12.5	56.3	12.5	18.8	0.0
Temperature + 38 ºC + magnetic pulse processing	56.3	0.0	50.0	18.8	37.5	6.3	81.3	12.5

*45 and 90 are the number of days of cultivation.

ranging from 81.3% (in medium containing ribavirin) to 93.8% (in medium containing salicylic acid + jasmonic acid). In the control treatment at the temperature of +20°C, the survival rate was 100%. Increasing cultivation duration to 90 days resulted in a decrease in survival rate by 12.5%–31.3%. In the control treatment with cultivation for 90 days, the explant survival was 75%. Explant death primarily occurred due to tissue intoxication and the accumulation of plant waste products in the nutrient medium.

By exposing explants to the temperature of 38 °C for 45 days and as compared to the standard cultivation temperature (+20 °C), resulted in a significant reduction in survival rate by using ribavirin (at 25%) and salicylic acid + jasmonic acid (at 37.5%). However, the exception was the salicylic acid treatment, where the reduction was only 6.2%. Consequently, salicylic acid increased the explants' resistance to the highest temperatures (38 °C) due to its anti-stress effects. In the variant without any treatment, the highest temperature resulted in 81.2% death of explants. By culturing apple dwarf rootstock explants at a temperature of +38 °C, the survival rate decreased by an average of 1.7 times compared with a temperature of +20 °C. Increasing cultivation duration to 90 days at the temperature of +38 °C resulted in complete explant mortality in the control, while survival in media containing AVD was low, ranging from 12.5% to 18.8%.

Exposure to high temperature combined with magnetic pulse treatment on a medium without AVD for 45 days ensured the highest explant survival (81.3%). However, with an increase in cultivation duration to 90 days, explant survival in this variant lowered to 12.5%. With the highest temperature (+38 °C) and MPP, the explant survival in media containing ribavirin and salicylic acid was 56.3% and 50%, respectively, while in media containing salicylic and jasmonic acids, the explant survival was lower (37.5%).

The integrated application of thermotherapy and MPP (without AVD) resulted in the highest survival rate (81.3%), while the combination of thermotherapy and salicylic acid also gave an 81.3% survival rate. The variants with a combined application of thermotherapy + ribavirin, thermotherapy + ribavirin + MPP, and thermotherapy + salicylic acid + jasmonic acid provided a moderate survival rate (56.3%).

In dwarf apple rootstock 62-396 explants, the growth parameters depended on the type of treatment, temperature, and magnetic treatment. Application of the studied treatments at a temperature of 20 °C after 1.5 months of cultivation resulted in a 2.1 to 3.2-fold reduction in shoot number compared with the control treatment (Table 2).

Increasing the temperature (+38 °C) inhibited the apple shoot proliferation in all treatments, reducing shoot number by 2.9 times in the control and 1.1 to 1.5 times in the variants treated with antiviral drugs. Additional treatment with magnetic pulse processing reduced the inhibitory effect of the highest temperature on shoot number in the control; however, it has no effect on shoot number in the variants treated with AVD.

Table 2. The number of shoots in the explants of the clonal rootstock of the apple tree 62-396 in the process of viruses' elimination, depending on the action of chemical and physical factors after 1.5 months of cultivation.

Physical factors of cultivation	Antiviral drug			
	Ribavirin (standard)	Salicylic acid	Salicylic acid + jasmonic acid	Control (without drug)
Temperature + 20 °C	1.8±0.4	1.5± 0.4	1.2± 0.2	3.8± 0.6
Temperature + 38 °C	1.4±0.2	1.0± 0.1	1.1± 0.2	1.3± 0.2
Temperature + 38 °C + magnetic pulse processing	1.2±0.2	1.1± 0.1	1.3± 0.2	1.9± 0.3

Table 3. Length of shoots in explants of the clonal rootstock of apple tree 62-396 in the process of viruses' elimination, depending on the action of chemical and physical factors after 1.5 months of cultivation (mm).

Physical factors of cultivation	Antiviral drug			
	Ribavirin (standard)	Salicylic acid	Salicylic acid + jasmonic acid	Control (without drug)
Temperature + 20 °C	15.3±2.1	10.4±1.6	9.7±1.4	21.9±3.3
Temperature + 38 °C	12.6±1.8	6.6±0.9	10.7±1.8	8.0±1.5
Temperature + 38 °C + magnetic pulse processing	9.4±1.2	8.6±1.1	9.1±1.3	12.2±2.0

*Different letters indicate significant differences at the 5% significance level.

Figure 2. Explants of apple clonal rootstock 62-396 after 90 days of therapy at a temperature of +20 °C, depending on the type of antiviral drug in the nutrient medium (from left to right): 1 – ribavirin, 2 – salicylic acid, 3 – salicylic acid + jasmonic acid, and 4 – control (no drug).

Similar trends were evident for shoot length and shoot number. The clonal dwarf apple rootstock 62-396 explants produced the longest shoots in the control, while the shortest shoots recorded emerged with the application of AVD at the highest temperature (+38 °C) (Table 3). At a temperature of +20 °C, the AVD inhibited growth processes in explants (Figure 2). With the temperature of 38 °C, MPP increased the shoot length by 1.5 times only in the control. Ribavirin treatment has a lesser inhibitory effect on growth processes than the variant with salicylic acid.

The explants exposure to combined applications of +38 °C and AVD and +38 °C + AVD + MPP, maximum shoot length occurred after the first transplant in the variant with the addition of salicylic and jasmonic acids (Figure 3). The yield of latent virus-free explants of clonal dwarf apple rootstock 62-396 in vitro

Figure 3. Explants of clonal rootstock of apple tree 62-396 at a temperature of +38 °C + MPP after the first transplantation to a medium without AVD, depending on antiviral drugs: at left – salicylic acid, at center – salicylic acid + jasmonic acid, and at right – control (without the drug).

Table 4. The yield of virus-free explants of apple clonal rootstock 62-396 in vitro depending on the action of chemical and physical factors.

Therapy option	ACLSV		ASGV	
	Yield of virus-free explants (%)	Infection index	Yield of virus-free explants (%)	Infection index
Control (without drug)	0.0	25.8±3.2	0.0	9.8±1.3
Ribavirin + thermotherapy (standard)	100	1.3±0.2	100	1.2±0.2
Thermotherapy + magnetic therapy	100	1.3±0.2	100	1.1±0.1
Salicylic acid + jasmonic acid + thermotherapy	100	1.6±0.4	50.0	3.7±0.5
Salicylic acid + jasmonic acid + thermotherapy + magnetic therapy	100	1.2±0.2	100	1.2±0.2

using various treatment methods varied from 50% to 100% (Table 4). A low virus infection index (1.2 for ACLSV and ASGV) was apparent with the complex therapy using AVD (salicylic acid + jasmonic acid), thermotherapy, and MPP.

DISCUSSION

The promising research revealed the duration of heat treatment significantly affected the survival of apple (*M. domestica* BORKH) explants. Increasing the cultivation duration to 90 days at +38 °C resulted in complete explant mortality in the control, while in media containing AVD, the survival rate was low, ranging from 12.5% to 18.8%. In experiments carried out by Iranian researchers, the percentage of surviving pear microshoots also relied on the duration of heat treatment. It decreased with increasing duration, and after 55 and 70 days, the survival rates were 83.3% and 33.3%, respectively (Karimpour *et al*., 2020). The increased explant mortality with increasing duration of heat treatment appeared to be associated with the active accumulation of plant metabolic products in the nutrient medium, including phenolic compounds. High temperature contributed to the increased enzyme activity, which leads to significant oxidative processes, the development of necrosis of leaves and shoots, and plant death (Vivek and Modgil, 2018).

Salicylic acid, both alone and in combination with MPP, considerably reduced the explant mortality with increasing cultivation duration for 90 days due to increased explant tolerance to the highest temperature for its anti-stress effects (Zhang *et al*., 2025). The explants' survival assessment suggested the feasibility of using antiviral agents at the cultivation temperature of +20 °C for 45 and 90 days. Increasing cultivation duration on media containing AVD could contribute to an increased yield of virus-free plants. Cultivating explants for 45 days with AVD at the temperature of +38 °C ensured a relatively high survival rate, while increasing the duration to 90 days led to a significant decrease.

The results further revealed the highest efficacy of ribavirin as an antiviral drug for treating the apple trees infected with ACLSV and ASGV, consistent with the findings obtained by other researchers. Ribavirin at the concentrations of 25, 50, and 100 mg/L resulted in 60%, 91%, and 100%, respectively, of Jonagold apple ACLSV-free regenerants. The combination of in vitro thermotherapy (+37 °C for three weeks) and ribavirin at concentrations of 50–100 mg/L resulted in complete elimination of ACLSV in apple regenerants (Cieslinska and Zavadska, 1999). However, effective concentrations of antiviral drugs can significantly inhibit growth processes in explants, often resulting in their death. A past study enunciated that ribavirin at concentrations of 75–100 mg/l caused severe (up to 100%) necrosis of apple tree shoots (Romadanova *et al*., 2021). Therefore, the study recommended reducing the concentration of ribavirin.

Iranian scientists have observed the high efficiency of pear recovery from ApMV and ASPV viruses (100%) with a combination of thermo- and chemotherapy (ribavirin 10–20 mg/l) in vitro (Karimpour *et al*., 2020). In China, the combination of thermotherapy (+36 °C) and ribavirin (25 mg/l) yielded 95% of apple explants free of latent viruses (Lizarraga *et al*., 2017). In Spain, during the recovery of six apple cultivars, the use of thermotherapy (+40 °C) in vitro followed by isolation of meristematic apices of 0.7–1.0 mm in size made it possible to obtain 100% of plants free from ApMV and 57%–100% free from ACLSV (Hu *et al*., 2015).

In the presented study, the combined application of thermotherapy and magnetic pulse processing ensured the highest yield of dwarf apple rootstock explants free of the studied viruses. The high effect of the combined use of thermotherapy and magnetic pulse processing may be because the simultaneous effect of two methods of healing from viruses leads to a more significant elimination of viruses. Consequently, it caused a decrease in their concentration compared with using just one method. In our previous experiments on pear, the yield of virus-free explants using various treatment methods ranged from 71.4% to 100%. The lowest virus infection index (1.2–1.3 for ACLSV and 1.2 for ApMV) with the highest yield of virus-free explants (100%) was notable with the use of salicylic acid and the combination of salicylic acid and heat treatment (Upadyshev, 2024). The use of magnetic pulse processing on apple and pear plants provided 86% of the explants recovery from the complex of viruses ASPV, ASGV, ACLSV, and ApMV (Upadyshev *et al*., 2021a, b, 2023, and 2025; Upadyshev and Makarov, 2025).

Previous studies on various crops have demonstrated the effectiveness of electrotherapy and magnetic therapy for virus treatment. For example, a virus treatment method for potatoes reached its development using a magnetic field with an induction of 3 to 5 Tesla and a pulse frequency of 4–51 kHz, resulting in a 67%–100% in vitro yield of PVS virus-free plants (Shevchenko *et al*., 2021). Electrotherapy of potato micro-cuttings resulted in 21% of plants being free of PVA and PVY viruses (Emami *et al*., 2011).

Electrotherapy of grape cuttings infected with the CLRaV virus in a constant electric field of 10 V/cm for 20 minutes, followed by planting the explants on a nutrient medium, resulted in the recovery of 33%–67% of regenerated plants. Meanwhile, chemotherapy with ribavirin resulted in the recovery of 67%–71% of virus-free plants (Guta *et al*., 2010). In this study, using the electrotherapy served as an alternative to thermotherapy. Overall, the present and past findings revealed the potential of using various healing methods (thermotherapy, chemotherapy, and magnetic pulse processing) to obtain the virus-free apple plants in vitro. The advantage of magnetic pulse processing over chemotherapy is its environmental safety.

CONCLUSIONS

With the cultivation temperature (+20 °C), establishing the possibility of using AVD for 90 days succeeded. In explants of dwarf apple (*M. domestica* BORKH) rootstock 62-396, the growth parameters depended on the type of AVD, temperature, and MPP. Increased temperature (+38 °C) inhibited the shoot formation in all variants. The yield of latent virus-free explants of the clonal dwarf apple rootstock 62-396 in vitro using various treatment methods ranged from 50% to 100%. A low virus infection index (1.2) was evident with the complex therapy using AVD (salicylic acid + jasmonic acid), thermotherapy, and magnetic pulse processing.

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