Elimination of *Garlic common latent virus* from garlic through meristem culture and thermotherapy

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Abstract. Virus infection on garlic (*Allium sativum*) can be accumulated through vegetative propagation and may cause yield losses. It was evidenced in this study that infection of GCLV on garlic seed cv ‘Tawangmangu Baru’ reached 100% based on detection using DIBA (Dot-immuno binding assay). This research was initiated to obtain an elimination method of GCLV from garlic seed bulbs of cv ‘Tawangmangu Baru’. Tissue culture-based method was used in this research involving isolation of 1.0 mm meristem from micro shoots followed by thermotherapy at 25 °C, 28 °C, 31 °C, and heterogeneous temperatures for 4 wk. Virus indexing was conducted using RT-PCR on micro shoots that grown after treatment. The results showed that meristem culture and thermotherapy did not affect the growth of micro shoots based on assessment on the percentage of surviving explants, plant height, and the number of leaves. Infection of GCLV was still detected on micro shoots after thermotherapy, i.e. 64% to 91%. Virus infection on garlic cv ‘Tawangmangu Baru’ was not totally eliminated by meristem culture and thermotherapy. Factors affecting this result will be discussed in this paper as well as methodology improvement to produce virus-free garlic with high multiplication rate of explants.

1. Introduction
Garlic is one of horticulture commodities widely used in Indonesia. The main central production areas of garlic are located in Java and Lombok. The farmers on those regions grow local cultivars such as ‘Sangga Sembalun’ in Lombok, ‘Lumbu Kuning’, Lumbu Hijau’, and ‘Tawangmangu Baru’ in Java. They generally use the bulbs harvested from previous season as seed sources and these garlic seeds is not certified. Garlic seed health is then becoming an issue since virus accumulation may occurred through seed bulbs. Virus infection of garlic in Indonesia have been reported, among others are GCLV (*Garlic common latent virus*), SLV (*Shallot latent virus*), and OYDV (*Onion yellow dwarf virus*) [1, 2].

Garlic is generally propagated vegetatively through bulbs and barely produces true seed through generative reproduction. Vegetative propagation may cause accumulation of viruses from one generation afterward that lead to the decreasing yields. It was reported that the productivity was decreased on garlic grown using virus-infected bulbs for 5 consecutive yr as well as its regeneration [3].
The control of virus infection on garlic is difficult if the disease has spread out in the field. Prevention of virus infection is an effective way in controlling plant viruses. The use of virus-free garlic seeds may reduce the initial inoculum, although reinfection will occur in the field [4]. The use of *in vitro* culture is a promising method to produce virus-free garlic seeds, especially when it is combined with other treatments such as cryotherapy, chemotherapy, electrotherapy, and thermotherapy. The adventitious bud culture combined with electrotherapy and thermotherapy was reported successfully eliminating OYDV with the efficiency reached up to 60% for garlic cv ‘Lumbu Hijau’ and 40% for cv ‘Sangga Sembalun’ [10].

The objective of this research was to confirm the incidence of GCLV in seed bulb cultivar ‘Tawangmangu Baru’ and develop *in vitro* culture-based method to reduce virus infection from garlic bulb.

2. Methods

2.1. Detection of GCLV on garlic seed bulbs

2.1.1. Sampling. Garlic seed bulbs cv ‘Tawangmangu Baru’ was obtained from seed breeder in Kalisoro Village, Tawangmangu District, Karanganyar, Central Java. As many as 50 cloves were taken randomly from a total of 1 kg of seed bulbs. Each clove was then grown in sterile husk charcoal medium for 2 wk. The leaves were then picked out as samples for virus detection using DIBA (Dot immuno-binding assay).

2.1.2. Virus detection using DIBA. Specific antibody to GCLV, i.e. GCLV IgG and IgG-AP (DSMZ) was used for virus detection using DIBA. A total of 0.1 g of leaf sample was grounded in Tris Buffer Saline (TBS: Tris-HCl 0.02 M and NaCl 0.15 M, pH 7.5) 1:10 (w/v) until leaf extract was obtained. The leaf extract was dripped onto the nitrocellulose membrane; the membrane was immersed in a blocking solution (TBS + 2% Triton-X) for 2 hr after the sample spot dried up. The membrane was removed and transferred to a container that contains IgG antiserum (in TBS + 2% skim milk) for overnight incubation at 4 °C, followed by incubation in IgG-AP (in TBS + 2% skim milk) for 2 hr at room temperature. The membrane is now ready for staining stage following washing using TBS containing 0.05% Tween-20. Membrane was incubated in substrate buffer (Tris-HCl 0.1 M, NaCl 0.1 M and MgCl₂ 5 mM) containing dye solution (Nitro Blue Tetrazolium + Bromo Chloro Indolil Phosphate). Positive reaction is indicated by colour change from white to purple on the nitrocellulose membrane containing the sample. The percentage of viral infection was calculated using the following formula:

\[
\text{Percentage of virus infection} = \frac{\text{Number of samples infected}}{\text{Total number of samples detected}} \times 100\%
\]

Virus titer in the sample was determined based on DIBA scoring on a scale of 0 to 4 (Fig. 1).

**Figure 1.** DIBA score based on the level of colour intensity on the nitrocellulose membrane; (0) no reaction occurs; (1) very weak; (2) weak; (3) strong; (4) very strong.

2.2. Virus elimination by meristem culture and thermotherapy

2.2.1. Meristem culture and thermotherapy. *In vitro* culture began with sterilization by washing cloves using antiseptic soap, followed by incubation in Dithane and Agrept (50:50) overnight on a shaker at 50 rpm. The sterilization stage was continued the next day under the laminar air flow, involving treatment using gradual concentration of chlorox during shoot excising. Shoots were excised...
gradually from the size 8 mm, 6 mm, and 4 mm with chlorox concentration of 15%, 10%, and 5% respectively. The final shoots (4 mm in size) were then planted on solid MS0 medium + BAP 0.4 ppm + GA3 0.4 ppm. After a week, meristem isolation was carried out from contaminant-free micro shoots. Isolation was done under a stereo microscope in aseptic conditions to obtain 1.0 mm meristem. These meristems were planted in liquid MS0 medium + BAP 0.4 ppm + GA3 0.4 ppm using filter paper bridges inside the bottle. Explants that survived were then given thermotherapy treatment. The culture room was prepared based on the need for temperature treatment, i.e. 25 °C, 28 °C, and 31 °C. For homogeneous temperature treatment, explants were grown directly at each temperature for 4 wk; while for heterogeneous temperature treatment, explants were grown at 25 °C, 28 °C and 31 °C for 1 wk consecutively and a final treatment at 31 °C for 1 wk. After 4 wk of thermotherapy, the micro shoots that survived were grown at ± 24 °C. Micro shoots were then used for virus detection using reverse transcription polymerase chain reaction (RT-PCR).

2.2.2. Experimental design. In vitro culture experiments was arranged in a completely randomized design with heat treatment factor at the level of 25 °C, 28 °C, 31 °C and heterogeneous temperatures. Each experimental unit was repeated 5 times, each replication consisted of 3 culture bottles, and each bottle contained 1 explant. The variables observed included the percentage of surviving explants, height of micro shoots, and number of leaves. Observations were conducted for 4 wk during treatment and 4 wk after treatment. Data was analysed using variance analysis (ANOVA) to determine the effect among treatments, followed by Tukey's analysis with 95% confidence interval if there were differences among treatments.

2.2.3. Virus Detection using RT-PCR. Total RNA extracted from the microshoots [5] were used as template in one step RT-PCR. The RT-PCR reaction consisted of 12.5 µL Go Taq Green (Taq DNA Polymerase (0.05 U/µL), reaction buffer, 2 mM MgCl2, dNTP 0.2 mM) (Thermo scientific), 1 µM each GCLV specific primer, 4 mM DTT, 0.1 µM RNAse inhibitor (Bioline) (40 units/µL), 0.1 µL reverse-transcriptase enzyme (Bioline) (200 units/ µL), 2 mM MgCl2, and 3.8 µL of nuclease-free water. Specific GCLV primers, i.e. GC-RT1 (5'-CGA AGC AAA CCA AGCAG-3') and GC-RT2 (5' CCC AGCAG-3 AGC AAA TTG ') [6] was targeted to amplify 306 bp specific DNA. Amplification was performed in GeneAmp PCR System 9700 with PCR protocol consisted of reverse transcription of RNA to cDNA at 45 °C for 20 min followed by pre-denaturation at 94 °C for 5 min and 29 cycles of denaturation at 94 °C for 1 min, primer attachment at 52 °C for 1 min, and elongation at 72 °C for 1 min; then ended with final elongation cycle at 72 °C for 10 min. DNA visualization was done using electrophoresis on 50 Volt for 50 min using 1% agarose gel.

3. Results and discussion

3.1. Symptom and frequency of GCLV infection
Observations were made on symptoms that appeared on 2 wk-old garlic plants. The main symptoms involved wrinkles on the surface of the leaves, yellow mosaic, green mosaic, and curves on the tips of the leaves (Fig. 2). These symptoms might be the results of GCLV infection mixed with other garlic viruses. It has been reported that mix infection of Carlavirus (GCLV, SLV), and Potyvirus (OYDV, SYSV) will result on yellowing and mosaic symptoms [7].

Confirmation of GCLV infection was carried out by DIBA using GCLV specific antiserum. Very high infection rate of GCLV (100%) was obtained from garlic samples, i.e. garlic seed bulbs grown on husk charcoal media with the average of DIBA scoring reached 2.58. It was reported previously that infection of GCLV together with Potyviruses reached 42.9% on garlic seed bulbs from Bandung, West Java causing yellow spot and wrinkle on the leaves surfaces [1]. Furthermore, virus detection on some other local garlic cultivars, including ‘Lumbu Hijau’, ‘Lumbu Kuning’, ‘Sangga Sembalun’, and ‘Tawangmangu Baru’, was reported [2]. It was shown that mix infection of GCLV and OYDV was
higher (43.5%), than single infection by GCLV (5.5%). All of these reports indicated that virus infection is a potential factor contributing to low yield of garlic production in Indonesia.

![Symptoms on young garlic plants at 2 wk after planting](image)

**Figure 2.** Symptoms on young garlic plants at 2 wk after planting; (a) Wrinkles; (b) yellow mosaic; (c) green mosaic; (d) curves on the tips of leaves. The symptoms were showed by the red arrows.

3.2. The effect of meristem culture and thermotherapy on the percentage of explants survived, the height of micro shoots, and the number of leaves

Thermotherapy at 25 °C, 28 °C, 31 °C and heterogeneous temperature did not affect significantly on the percentage of surviving explants. During the 4 wk of thermotherapy, the number of explants that survived began to decline in the second wk to the third wk (Fig. 3a). The death of explant might be caused by its ability to survive in high temperature. The higher the temperature, the more the number of explants failed to grow. It has been reported that the explants subjected to treatment of 37 °C suffered deaths up to 30%, while at 40 °C and 45 °C, explant deaths increased up to 80% and 100% [8]. Additionally, the explants’ resistance to high temperatures was influenced by explant size [9]. At 4 wk after thermotherapy, the number of explants that survived declined (Fig. 3b). Explant death in this period was related to the availability of nutrients in the growth medium. Sub culturing to new media should be undertaken after 4 wk so that the availability of nutrients was sufficient for explant growth.

![Percentage of explants survived](chart)

**Figure 3.** The number (%) of garlic explants that survived after thermotherapy at different temperatures (25 °C, 28 °C, 31 °C, and heterogeneous). Four week during thermotherapy (a) and 4 wk after thermotherapy (b).
Thermotherapy also has no significant effect on the height of the micro shoots. The highest increase in shoot height occurred at 25 °C treatment, while the lowest occurred at 31 °C treatment, both for 4 wk during treatment (Fig. 4a) and 4 wk after treatment (Fig. 4). The treatment of thermotherapy may cause a risks of inhibiting the regeneration of explants and even cause death. Previous experiment showed that the adventitious bud culture of garlic cv ‘Lumbu Hijau’ and 'Sangga Sembalan' could not grow in the treatment of electrotherapy combined with thermotherapy at 38 °C, and the increase in plant height was inhibited at 33 °C [10]. In vitro culture with liquid medium, temperature treatment above 28 °C causes medium evaporation then resulting in water vapour formation on micro buds and culture bottle thus it can inhibit bud growth [11].

![Figure 4](image1.png)

**Figure 4.** The average of micro shoots height (cm) of garlic explants at different temperatures (25 °C, 28 °C, 31 °C, and heterogeneous); (a) 4 wk during thermotherapy; (b) 4 wk after thermotherapy.

The increase in the number of leaves was not significantly affected by the thermotherapy treatment. The increase in the number of leaves at all temperature treatments began in the first wk but the number of leaves did not increase after the third wk (Fig. 5a). The decrease of the number of leaves after treatment occurred in the fifth to eighth wk (Fig. 5b). This condition can be caused by the senescence phase which is experienced by micro shoots, where the leaves will turn white, and dry out, finally cause shoots death [9].

![Figure 5](image2.png)

**Figure 5.** The average numbers of leaves of garlic explants at different temperatures (25 °C, 28 °C, 31 °C, and heterogeneous); (a) 4 wk during thermotherapy; (b) 4 wk after thermotherapy.
3.3. Virus incidence on micro shoots after thermotherapy

Specific DNA bands of GCLV was amplified from several samples of micro shoots grown from meristem culture method combined with thermotherapy (Fig. 6). It indicated that GCLV still presents in some micro shoots. Virus incidence reached 92%, 91%, 64%, and 67% in samples given 25 °C, heterogeneous, 28 °C, and 31 °C treatment, respectively (Table 1).

The efficiency of meristem culture and thermotherapy for virus elimination can be related to several things including changes in morphology, physiology, and plant resistance. The meristem part with the size of < 1.0 mm is relatively virus-free because in this section, plasmodesmata that is known to be the pathway of virus cell-to-cell movement have not been formed. In addition, heat treatment could expand the plant cells then suppress the plasmodesmata size so that it could inhibit virus movement. Thermotherapy can also activate plant defenses against viruses through RNA silencing mechanisms [12]. However, meristem culture and thermotherapy do not always succeed in eliminating the virus, although in many cases it is able to reduce its concentration. It was reported that meristem culture and thermotherapy produced 38.8% of GCLV-free plantlets, the lowest when compared with OYDV (83%) and LYSV (100%) [13]. GCLV was known as the most detectable virus in leaves, bulbil, and plantlets in vitro. The virus was also the most persistent in plantlets in vitro compared to other viruses such as OYDV, LYSV, SLV, and MbFV [14].

| Treatment         | Infection of GCLV (%) |
|-------------------|-----------------------|
| 25 °C             | 92 (11/12)³           |
| 28 °C             | 64 (7/11)             |
| 31 °C             | 67 (6/9)              |
| Heterogeneous     | 91 (10/11)            |

³Numbers in parentheses indicated the number of GCLV infected shoots / total number of shoots detected.

![Figure 6. Agarose gel electrophoresis (1% agarose) of RT-PCR amplified product using specific primers for GCLV (GC-RT1/-RT2). M, DNA marker 100 bp; N, negative control; P, positive control; 1-4, 5-8, 9-12, and 13-16 samples from thermotherapy at 25 °C, 28 °C, 31 °C, and heterogeneous temperature, respectively.](image)

4. Conclusion

The incidence of GCLV was found in garlic seed bulbs cv ‘Tawangmangu Baru’. The use of seed bulbs originating from the previous planting season may contribute to the incidence of viruses in the field. The combination of meristem culture technique and thermotherapy has the potential to eliminate
GC LV on the micro shoots cv ‘Tawangmangu Baru’. The infection was successfully suppressed by 9 to 46% after the heat treatment.

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