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Thermotherapy of cloves for *in-vitro* mericlone production for healthy and sustainable management of garlic (*Allium sativum* L) germplasm

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Garlic is a second most important crop of the genus *Allium*, known for its traditional medicinal uses. In spite of having medicinal prosperities, it gets infected by several viruses called Garlic Viral Complex (GVC). The garlic viral complex severely affects bulb yield of garlic, due to accumulation of virus load year after year from asexual propagule cloves. Hence we attempted an experiment to eliminate garlic viral complex through thermotherapy in combination with meristem culture. The viruses were eliminated from infected garlic bulb with the treatment at 37°C for 90 days followed by meristem culture could eliminate the Garlic Common Latent Virus, Onion Yellow Dwarf Virus and Allexiviruses. In this study, we confirmed that the combination of thermotherapy and meristem culture technique protocol have wider prospects for virus elimination in garlic.

Keywords: Bulb, Clove, Garlic, GVC, Meristem culture, Thermotherapy

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Garlic (*Allium sativum* L) is an important traditional medicinal plant considered as a spice as well as vegetable, it plays a very crucial role in the health of human being due to the medicinal properties and it helps in prevention of various diseases such as diabetes, asthma, cardiovascular diseases, and cancer. The garlic traditionally being used in the culinary purposes due to its unique aroma, flavours and bioactive compounds such as Allicin (allyl2-propenethiosulfinate or diallylthiosulfinate), oligosaccharides, essential oil, steroidal glycosides, anthocyanins, flavonoids, lectins, prostaglandins, pectin, fructan, adenosine and vitamins has highly valued medicinal and nutraceutical properties[1-3]. The centre of origin of garlic revealed to be Central Asia, where more diversity of garlic was found. Commercially cultivated garlic is diploid (2n=16), sexually sterile, do not produce seeds through sexually and does propagate through clonally from the era of antiquity, although some hard necked garlic germplasm were fertile and which has limited scope for exploitation for commercial cultivation through seeds due to climatic preference of bolting and seed production. Since, the beginning of human civilization and the industrial revolution, human activities have bought about tremendous changes in atmospheric composition and climate. Unpredictable climate change poses great challenges for the survival of various *Allium species* and their landraces.

Asexually propagation of garlic easily leads to infection of a mixture of viruses *Potyviruses* and *Carlaviruses* and *Allexiviruses*[4]. Virus infection significantly reduces the potential of garlic yield because garlic is vegetatively propagated crop[5,6]. The severe yield losses were caused by *Potyviruses* namely Leek Yellow Stripe Virus (LYSV), Onion Yellow Dwarf Virus and Iris Yellow Spot *Tospovirus*[7]. The sixteen viruses of genus *Allexivirus*, *Potyvirus*, *Potexvirus*, *Carlavirus* and *Tospovirus* are problematic in garlic production and the viruses collectively called as Garlic Viral Complex (GVC). Garlic *Carlavirus*es namely Garlic Common Latent Virus (GarCLV, or GCLV), Shallot latent virus (SLV) and Mite–Borne Mosaic Viruses (*Allexiviruses*), *Potyviruses* namely Onion Yellow Dwarf Virus (OYDV) and Leek Yellow Stripe Virus (LYSV) invasion cause loss of 50% in garlic bulb yield[8]. *Allexivirus* namely GarMbFV infestation reduced the

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bulb yield of 32% in the first year upon artificial virus infestation. A cumulative garlic bulb yield loss noted 50% through Garlic Viral Complex[9]. These garlic bulb yield losses were due to poor breeder seed replacement for commercial production; it is attributed by non-availability of the authentic virus-free seed cloves, asexually propagation of planting material results in accumulation of virus load in the clove, thereby increases bulb yield losses year after year. Hence, it is a prime need to ensure that the garlic clove planting material is free of viruses. This is could be possible through thermotherapy and meristem culture. The indigenous traditional knowledge for reducing the virus load was commonly followed by farmers in terms of treating garlic bulb immediately after harvest in the sunlight for field curing of garlic bulb, this techniques might also involve in cleaning of virus-infected plants by reducing the virus replication rate under higher temperature furthermore meristem culture of these thermotherapy subjected cloves assured to be free from the viruses load. The virus-free garlic planting material has the potential to increase yield and further supplement sustainable management[10].

Thermotherapy is a method in which plants or plant organs were treated for temperature between from 35°C to 54°C for a specific period without altering of their physiological tolerance limit. Temperature range and time duration are standardized based on the rate of virus degradation and the survival rate of explants, cloves with meristematic tissues had undergone thermotherapy resulted in the generation of fewer plantlets than the non–treated meristematic cloves, 90 to 100% plants from thermotherapy were found to be free from OYDV[11]. The 90% of plants were free of the virus in garlic cv. Amarante was obtained through dry-heat thermotherapy (37°C for 35 days)[12]. However, though mericlones raised through thermotherapy were found to be free from viruses load, but the overall virus elimination efficiency was low[13]. Thermotherapy at the temperature ranged from 30°C to 36°C for 4 and 5 weeks in dry chamber followed by the stem–disc–dome culture resulted in 61 and 77% elimination of viruses GarVs and OYDV, respectively[14]. Thermotherapy in combination with meristem culture resulted in 100% elimination of LYSV significantly than shoot tips culture and meristem culture alone[15].

Meristem culture is a renowned and popular technique mainly used to isolate virus-free plants. Virus elimination by meristem culture is based on the act of fact that these meristematic cells were free from viruses due to higher rate of cell division, which is faster in the meristematic tissue compared to the viruses. Therefore, plants were regenerated through meristematic tissue are virus-free. Messiaen and co–workers[16] were pioneers to regenerate garlic plants through meristem culture in 1970. Since then meristem culture has been used to produce virus-free garlic and other plant species. The plant regeneration through meristem culture is related to the size of meristem and its size will be the fate of obtaining virus-free plants, the virus-free plantlets production rate is inversely proportional to the meristems excised[17-21]. The 100% virus-free garlic plantlets were obtained by meristem culture from MS medium with 0.5 mg/L of 2–isopentenyl adenine (2–iP), NAA at 0.2 mg/L and sucrose of 30 g/L, as well as in MS medium with 2 mg/L of BA, indole–3–butyric acid (IBA) of 0.5 mg/L and sucrose of 30 g/L[22]. The virus-free plants were isolated from inflorescence meristem, bulbils and roots than apical meristem[23]. Meristems of size ranged from 0.5 to 1 mm were obtained from the cloves and bulbils, thus garlic plants reported to be 38% of explants from cloves and 25% from bulbils were free from viruses[24]. Considering the above facts and limitations in obtaining virus-free plants in garlic, the current study is mainly focused on optimization of thermotherapy of cloves before meristem excision on the regeneration and survival efficiency of garlic (bulbs). Further, based on viral gene amplification tests, one could predict the potential of the thermotherapy treatment for developing virus-free plants. The study will be proved to be useful for cultivation, management and conservation of garlic varieties and germplasms.

Materials and Methods

Plant material

Garlic variety Bhima-Purple was used in this study; it was developed and released for commercial cultivation by ICAR-Directorate of Onion and Garlic Research, Pune. The ICAR-DOGR variety Bhima-Purple has unique characteristics feature and commercially cultivated in various part of the country[25]. The 3 kg bulbs were collected during the *rabi* season 2016-17 were cure in the field for 48 hours and 15 days shade curing. Garlic clove was treated at 37ºC for 30, 45, 60, 75, 90 days 500 g of garlic bulbs were subjected for each thermotherapy treatments in hot-air-oven, regeneration and survival

rate were measured and isolated meristem and mericlones were established in *in-vitro*. Furthermore regenerated micro-bulbils and directly transplanted into the field and developed good quality of the bulb.

Isolation of meristem from the Garlic cloves

The meristems (~0.1 mm) were dissected from each treated clove under the microscope in laminar airflow and isolated. The meristems were transferred on Petri plate containing MS media 0.1 mg NAA and 1 mg BAP with 3% sucrose with 0.6% agar under laminar flow for direct regeneration of mericlones. The ex-plant containing Petri plates were subjected for culture in culture room maintaining 16000 lux of light, 18 h of light and 6 hours of the dark at 24ºC of temperature.

Induction of *in-vitro* shooting and developed bulbils, establishment bulbils in *ex-vitro* condition

The mericlones were sub-cultured for shooting and bulbing in *in-vitro* Murashige and Skoog media supplemented with 0.1 mg/L NAA and 1 mg/L BAP with 3% sucrose and 0.6 % agar were used for plantlet development. After 60 days the plantlets were subcultured for development of micro bulbils in MS medium with 10% sucrose and 0.6% agar for 45 days, cultures were maintained in 16000 lux of light, 18 hours of light and 6 hours of the dark at 24ºC of temperature. The *in-vitro* bulbils were harvested and then planted in the field for assessment of bulb yielding attributed during *rabi* season 2017-18.

Virus indexing by PCR amplification

Total RNA was extracted out of 100 mg tissues of mericlones of garlic plantlets, using RNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's protocol. The multiplex RT-PCR was performed with the use of previously reported primer and multiplex RT-PCR[26]. The cDNAs for OYDV, GarCLV and allexiviruses were prepared, the multiplex PCR was performed with reaction mixture having 5 µL of reverse transcription mixture, *Taq* DNA polymerase of 5 U (Promega, Madison, USA), 5 µL of 10× reaction buffer, 1.5 mM of $MgCl_2$, primers of 0.2 µM and 0.2 mM dNTPs. The temperature profiling with denaturation at 94ºC for 5 min, 30 cycles of 45 s at 94ºC, 20 s annealing temperature of 57ºC and 1 min at 72ºC and one final extension step at 72ºC for 10 min on a gradient PCR[26].

Results and Discussion

In this study, we optimized the exposure of thermotherapy to study the effect on survival and reducing the virus load on garlic plants. The results of the studies reveal that the temperature 37ºC for 90 days of thermotherapy could regenerate 25% of mericlone plantlets with highest time duration of 61 days (Table 1), which were the least in the per cent number of plant survival rate, but it has highly strong and potential for isolation of virus-free garlic plant, in which eliminated the Garlic Common Latent Virus, Onion Yellow Dwarf Virus and *Allexiviruses* (Fig. 1).

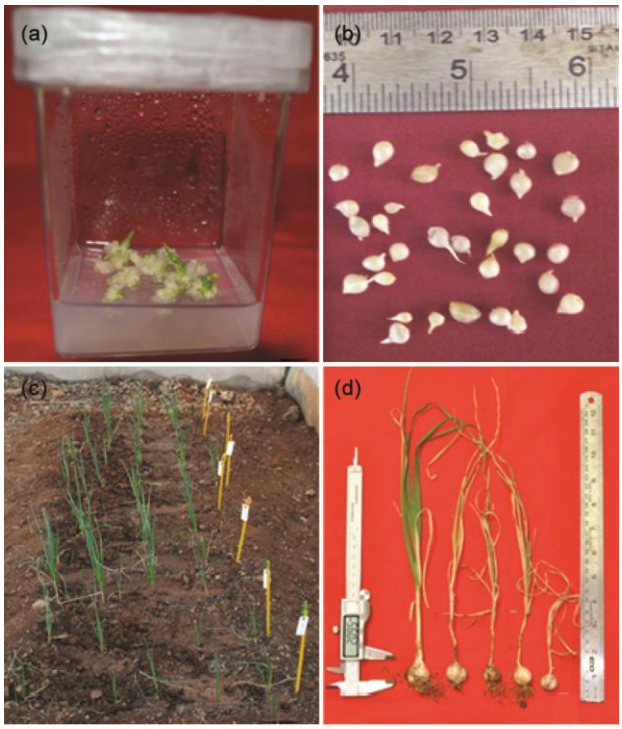

Fig. 1 — *In-vitro* garlic production of mericlones and their establishment in *ex-vitro*, A) regeneration of sub-cultured meristems, B) micro-bulbils developed in *in-vitro*, C) field establishment mericlone plantlets through miro-bulbils, and 4) Development of Garlic Bulbs in the field.

Table 1 — Effect of thermotherapy on survival rate and regeneration duration of mericlones

Treatments	Meristems isolated (No)	Meristems Survived (No)	Survival rate (%)	Regeneration time (days)
Control (No thermotherapy)	60	57	95	30
Thermotherapy for 30 Days at 37ºC	60	48	80	35
Thermotherapy for 45 Days at 37ºC	60	45	75	45
Thermotherapy for 60 Days at 37ºC	60	41	68	48
Thermotherapy for 75 Days at 37ºC	60	36	60	58
Thermotherapy for 90 Days at 37ºC	60	15	25	61

Table 2 — *Ex-Vitro* establishment of mericlone plantlets and micro-bulbils

Planting material	Total Plant transplanted (No)	The total plant survived (No)	Survival rate (%)
Mericlones plantlets	60	8	13.33
Mericlone micro-bulbils	60	35	58.33

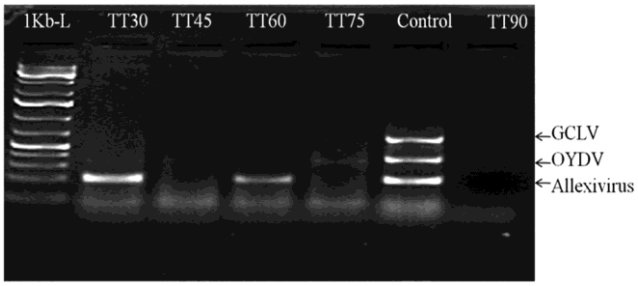

Fig. 2 — Virus indexing of mericlones at the differential treatment of thermotherapy

The percentage of the *ex-vitro* establishment of mericlones bulbils or micro-bulbils (58.33%) were greater than the mericlone plantlets (13.33%) (Table 2). Our study proved that long-duration heat treatment of garlic cloves (90 days 37ºC) have potential to develop a virus-free plant that could regenerate, survives, developed good quality bulb when micro-bulbils would be planted in field (Fig. 2). Similar studies were supported that the heat therapy treatment at 37ºC for 21 days before meristem excision produce 29% of GarCLV, SLV and OYDV[21,24,27]. The plants underwent thermotherapy or temperature elevation before meristem excision, normal development of the plant is affected and significantly lower number of shoots regenerated on a single meristem as compared to control[14,20,28,29]. Torres *et al*.[12] reported that thermotherapy reduced survival and regeneration rate but the rate of recovery of the virus-free plant was high further confirmed our study and hypothesis that higher duration thermotherapy treated clove have more potential to be garlic virus free. Our study reveals the methodology which could help in the regeneration and development of higher percentage of virus-free plants. Furthermore, mericlone micro-bulbils developed *in-vitro*, thereafter which were developed good quality bulb in the field as compared to tissue culture plantlets raised directly transplanted in the field. Most of the directly transplanted tissue culture plant died and unable to produce bulb compared to micro-bulbils transplanted plant which developed good quality of bulbs.

Conclusion

Though there is a research gap in the optimisation of temperature with time duration for successful development of a higher percentage of virus-free plants. Our study reveals that 37ºC for 90 days of thermotherapy treatment has the potential to develop a higher percentage of the virus-free plant. The thermotherapy in combination with meristem culture is suitable for the conservation and sustainable management of virus-free garlic. Further, the mericlone bulbils were able to develop good quality bulb with multiple cloves in the field. So this protocol with optimum temperature with suitable time duration in combination with meristem culture would help for the lab to land transfer of *in-vitro* virus-free garlic in commercial garlic sustainable bulb production.

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Conflict of Interest

We the authors declare no competing interest with this manuscript.

Author Contributions

DCM & KJ: Conceptualization, Experimentation, Formal analysis, Original draft; MS: Resources; VJ: Conceptualization; MS, RS, PC &VJ: Review & Editing of Manuscript.

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