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Virus–Free Seed Production of Garlic (*Allium sativum* L.): Status and Prospects

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A B S T R A C T

Garlic (*Allium sativum* L.) is a sterile diploid species, which do not flower and hence forms no seeds. Thus, it is asexually propagated by vegetative means of cloves, due to vegetative propagation of garlic is lead to infect by the several viruses, which are together called as garlic viral complex (GVC). The viruses go on accumulating in planting seed material due to continuous use of same source of viruses–infected true garlic seed (TGS) propagules year after year namely garlic cloves. GVC is the most important factor for lower yield of garlic in tropics. Thus, production of virus–free garlic seed is an important agricultural input to improve the yield of garlic. In this paper we review status and prospects of research in the area of virus–free garlic seed production.

Introduction

Garlic (*Allium sativum* L.) is a monocotyledonous diploid (2n = 16) plant belonging to genus *Allium* of the family Alliaceae. It is the second most widely distributed species of *Allium* after *A. cepa* (onion) (Kamenetsky, 2007). It is grown as vegetable condiment in major parts of the world. Central Asia is considered its center of origin (Vavilov, 1926). The discovery of fertile clones which produce true seeds of garlic (TSG) and primitive types of garlic in the region of Tien–Shan–mountains in Kyrgyzstan (Etoh, 1986; 1997, Kotlinska *et al.,* 1991) confirmed this. It was further supported by biochemical and molecular studies (Pooler and Simon, 1993). Garlic bulbs are composed of aggregate of cloves, which originate from axillary buds. The cloves are covered with thin protective sheath, which is dry at maturity, which were cover the thickened storage leaf sheath and foliage leaves by protecting the apical meristem (Mann, 1952). Number of cloves per bulb varies with the cultivar and ranges from 10 to 25 with an average of 16 cloves per bulb. Garlic is propagated vegetatively through cloves or top–sets developed from plant inflorescences. Garlic has low flowering ability (Kamenetsky and Rabinowitch, 2001; Etoh and Simon, 2002) and is sterile. The

sterility of the garlic is mainly attributed to chromosomal deletions, differences in the length of homologous chromosome, loss of genes involved in gametogenesis, hypertrophy of the tapetal layer of the anthers at the post–meiotic stage, microspore degeneration before and after the tetrad stage and nutritional competition between the topsets and flowers (Novak, 1972; Etoh, 1985; Pooler and Simon, 1994). However, true seeds of garlic (TSG) are reported by Etoh (1997) in few fertile clones of garlic, further true seeds were used in garlic improvement.

Garlic is successfully grown worldwide from temperate to sub–tropical climate (Fritsch and Friesen, 2002). The area and production of garlic have been increasing year after year all over the world. China is the largest producer of garlic in the world with production of 20.08 million ton from 0.86 million hectares followed by India, which has production of 1.25 million ton from 0.23 million hectares (Mamta, 2014; FAOSTAT, 2012). The productivity of garlic is low, because of this low productivity of garlic, stakeholder are not much interested in garlic cultivation. The main reason for its low productivity is non–availability of good quality planting seed material, lack of high yielding varieties which are free of viruses for cultivation in tropical and sub–tropical parts of world. Where, garlic is a short duration crop as compared to the temperate counter parts, could be other major factors limiting the garlic productivity.

Garlic being asexually propagated is easily infected by mixture of viruses including aphid transmitted *Potyviruses* and *Carlaviruses*, mites transmitted *Allexiviruses* (Cafrune *et al.,* 2006). The yield losses caused by *Potyviruses* are severe, leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV) and iris yellow spot tospovirus (IYSV) and are not transmitted by true seeds (Kritzman *et al.,* 2010). Almost sixteen major viruses of genus *Potyvirus, Allexivirus, Carlavirus, Potexvirus* and *Tospovirus* are reported in garlic, collectively called as garlic–viral–complex (GVC). Garlic common latent virus (GarCLV, *Carlavirus*), Shallot latent virus (SLV, *Carlavirus*) and mite–borne mosaic viruses (*Allexiviruses*), Onion yellow dwarf virus (*Potyvirus*) and Leek yellow stripe virus (LYSV, *Potyvirus*) infestation caused 50% bulb yield loss (Lot *et al.,* 1998). *Allexivirus* viz. GarMbFV reduced the yield up to 32% in first year after virus infestation of healthy plants (Perotte *et al.,* 2010). A cumulative yield loss up to 50% by GVC has been reported (Perez–Moreno *et al.,* 2010). Poor seed replacement rate and virtually nil availability of authentic virus–free planting seed material result in buildup of virus load in the planting seed material and thereby increased productivity loss year after year. Hence, it is very essential to ensure that planting seed material is free from viruses. This is possible through *in–vitro* tissue culture techniques like micro–propagation, meristem culture, thermotherapy, chemotherapy, cryotherapy and somatic embryogenesis. These techniques involve in cleaning of virus infected plants and their subsequent multiplication for virus–free propagules, which can be mass multiplied by growing in virus–free areas. The *in–vitro* approaches that can be used for production of virus–free seed in garlic are reviewed below.

In–vitro virus–free garlic seed production techniques

Micropropagation

It is a rapid method of *in–vitro* multiplication of organized tissues. Kamenetsky *et al*., (2015) studied the distribution of four *Allexiviruses* are garlic virus–A, garlic virus–C, garlic virus–E and garlic virus–X in six organs of garlic namely basal plate, leaves, inflorescence, flowers, roots and cloves. They

found differential viral transcript expression levels in different tissues using transcriptome analysis in virus–free and virus–infected garlic plants. The highest amount of virus RNA was observed in cloves (32–41%) and roots (32–50%), whereas least amount of transcript was recorded in leaves (1–4%), basal plate (5–7%), inflorescence (8–20%) and flowers (1–7%). Thus, the study revealed that for the production of virus–free garlic seed, the flowers, inflorescence, leaves and basal plate are ideal as these had reduced viral load. Stem disc culture in garlic results in the formation of roots, shoots and bulbs. Abo–El–Nil (1977) started garlic culture from stem and leaf discs of variety Extra–Early–White, resulting in the formation of adventitious multiple shoots (Osawa *et al.,* 1981). Micro–propagation of garlic varieties namely Isshuwase, Isshu–gokuwase, Shanhai, Santo, Furano and Howaito–roppen carried out by Nagakubo *et al.,* (1993) by shoot tip culture in the Murashige and Skoog (MS) medium containing 1–naphthaleneacetic acid (NAA, 1 µM) and benzyl adenine (BA, 1 µM). The scape tips of garlic cultured on MS medium containing NAA (2.6 µM) and kinetin (2.3 µM) led to formation of adventitious shoots and developed into plants which were free of garlic mosaic virus (GMV) (Ma *et al.,* 1994).

Ayabe and Sumi (1998) cultured stem disc containing apical meristem and lateral buds of clove to regenerate plants of garlic cultivar Fukuchi–Howaito. Cloves were cut into various fragments and cultured, as a result 20–25 adventitious shoots were obtained. Ayabe and Sumi (2001) cultured stem–disc to eliminate viruses from infected garlic plants. They could produce about 25 shoots on stem disc clove, maintained them on phyto–hormone free Linsmaier and Skoog (LS) medium. Plants were rooted after eight weeks of inoculation and successfully established in the soil. These were tested by direct tissue blotting immune assay and reverse transcription polymerase chain reaction which were free of viruses. Khan *et al.,* (2004) regenerated adventitious shoots from calli developed from root tips.

Meristem culture

Meristem culture is a popular renowned technique used to obtain virus– free plants in numerous vegetatively propagated crops. Virus elimination through meristem culture is based on the fact that these meristematic cells are almost free from viruses due to high rate of cell division, which is faster in the meristematic tissue than the viruses. Therefore, plants regenerated from meristematic tissue are virus–free. Messiaen and his coworkers were first to regenerate garlic plants from meristems in 1970. Since then meristem culture has been used to produce virus–free plants in various plant species of the world (Bhojwani *et al.,* 1982; Pena–Iglesias and Ayuso, 1982; Bertacinni *et al.,* 1986; Walkey, 1987; Conci and Nome, 1991). Important pioneer works in the field of garlic meristem culture are presented in table 1. Although, *in–vitro* regeneration directly proportional to the size of meristem and its size is a fate of obtaining virus–free plants, rate of virus–free plants production is inversely proportional to the excised meristem (Faccioli and Marani, 1998)

More virus–free plants are generated from inflorescence meristem, bulbils and roots than the apical meristem (Appiano and D'Agostino, 1983). Verbeek *et al.,* (1995) cultured meristems obtained from cloves and bulbils (0.5–1 mm), reported 38% of explants from cloves and 25% from bulbils contributed to the production of virus–free garlic plants. Xu *et al.,* (2001) regenerated virus–free plants from meristems obtained from inflorescences.

The various stages of growth, maturation and bulb development *in–vitro* are illustrated in

figure 1, which were obtained at Directorate of Onion and Garlic Research (DOGR) by meristem tip culture of garlic variety Bhima Purple. In this protocol, cloves were surface sterilized by dipping in bavistin (0.1%) containing streptomycin (0.02%) solution for 10–15 minutes followed by treated with 70% ethanol for 5 minutes, then cloves were dipped for 15 minutes in 2% sodium hypochlorite in laminar air flow chamber. After washing of cloves with sterile distilled water, meristem tips of 0.2–0.4 mm size were excised under laminar air flow with use of stereo–binocular microscope. These were inoculated on MS media containing NAA (0.1 mg/L) and kinetin (1 mg/L).

Cultures were incubated in growth room at temperature of 25 °C with light intensity of 10 000 lux for 16 hours light and 8 hours dark (unpublished data, DOGR, Pune).

Other media have been used in some laboratories for meristem culture are, Nagakubo *et al.,* (1993) used LS medium with 1 µM indole–3–acetic acid (IAA) and 1 µM BA for meristem culture. They could induce multiple shoots on modified LS medium containing 56.5 mM KNO_3 and 3.5 mM NH_4Cl, supplemented with 5 µM NAA and 10 µM BA. Ravnikar *et al.,* (1993) cultured meristems on Gamborg (B5) medium containing 1 mM IAA and 1 mM BA. After six weeks these were sub–cultured on medium containing 5 mM jasmonic acid (JA) and 5 mM 2iP.

The shoots obtained were multiplied in *in–vitro* and *ex–vitro,* indexed for onion yellow dwarf virus by DAS–ELISA test which were virus–free. Hatjra *et al.,* (2013) used MS medium containing 0.5 ppm 2iP with 0.2 ppm NAA and 3% sucrose or 2 ppm BA with 0.5 ppm IBA and 3% sucrose are used for meristem culture.

Thermotherapy

Thermotherapy is a process in which plants are treated at temperature between 35 °C to 54 °C for specific period of their physiological tolerance limit. Temperature and duration are standardized based on the virus degradation and survival rate of plants (Spiegel *et al.,* 1993). Meristem tissues that had undergone thermotherapy resulted in regeneration of fewer plants than the non–treated meristems, but 90 to 100% plants from thermotherapy treated meristem tissues were found to be free from OYDV.

Torres *et al.,* (2000) recovered 90% plants free of virus from the cloves of garlic cultivar Amarante through dry heat thermotherapy (37 °C for 35 days). However, according to Ramirez *et al.,* (2006) though mericlones produced through thermotherapy were found to be free from viruses, but the overall efficiency of virus elimination was low. Thermotherapy at 30 ^{o}C for one week and 36 ^{o}C for 4 and 5 weeks in dry chamber followed by stem disc dome culture resulted in 61 and 77% elimination of viruses GarVs and OYDV, respectively (Fahimeh *et al.,* 2014). Thermotherapy with meristem culture resulted in 100% elimination of LYSV significantly than shoot tips culture and meristem culture alone (Vieira *et al.,* 2015).

Chemotherapy

Since the discovery of ribavirin, chemotherapy is well known for virus elimination (Sidwell *et al.,* 1972; Huffman *et al.,* 1973). However, in plant virology due to less resource availability and delayed knowledge of molecular characteristics of many *phytoviruses,* the chemotherapy studies were limited. In garlic, Senula *et al.,* (2000) eliminated OYDV and LYSV through chemotherapy. Virus elimination *per se* increased by 85–95% using chemotherapy.

Sidaros *et al.,* (2004) used virazole for chemotherapy. The maximum virus–free plants (100%) were obtained when meristems of 0.3 mm size were cultured on MS medium with 50 mg/L virazole. Ramirez *et al.,* (2006) reported *Potyvirus* free plants by chemotherapy of cloves with 205 µM ribavirin with a survival rate of 27–34.8%. Ramirez–Malagon *et al.,* (2006) used thermotherapy, chemotherapy and meristem culture in combination to obtain *Potyvirus* group free garlic plants. Embryos were obtained from cloves which had undergone chemotherapy and the plants regenerated from embryos were given thermotherapy treatment. Meristems from these were cultured and plants obtained were indexed through ELISA. Thermotherapy was found to have negative effect on plants establishment. But thermotherapy in combination with meristem culture proved to be more efficient method for virus elimination (60 to 70.9%) than meristem culture (64%) alone. However, Ramirez–Malagon *et al.,* (2006) reported that chemotherapy was not effective for *Potyvirus* group elimination.

Cryotherapy

Cryotherapy is a the technique in which *ex*–plants are treated under ultra–low temperatures, usually at –196 °C with cryo agents especially liquid nitrogen, and this technology had been applied to eradicate the viral complexes of several plant species (Brison *et al.,* l997). However, Cryotherapy of shoot tips has became a greater prominence to eradicate plant pathogens and it is extensively could use, since cryopreservation protocols already well established in economically important vegetatively propagated crop species (Wang *et al.,* 2009; Wang and Valkonen, 2009). In cryotherapy, ultra–low temperature (–196 °C) revealed to be eliminate the pathogen of infected cells due the lethal effect of cryo–agent cause cell damage resulted in increased vacuolization of cells (Wang and Valkonen, 2008; Vieira *et al.,* 2015) this could lead to the low number of survival plants through *in–vitro* culture of ex–plants. However, cryotherapy could make easy to ex–plants excision, lead to the high proportion of virus–free plants regeneration. altogather, the cryotherapy could reduces the need of indexing to identify virus–free plants (Wang *et al.,* 2008; Wang and Valkonen, 2008) The shoot tips cryotherapy with thermotherapy resulted in insignificant survival of *ex*–plants. Combination of thermotherapy, cryotherapy with shoot tip meristem cultures could eliminate the OYDV, GCLV and LYSV (Vieira *et al.,* 2015). Cryotherapy eliminates plant pathogens such as namely viruses, phytoplasma and bacteria upon shoot tips treatment with liquid nitrogen. However, cryotherapy applied in large samples of their independent of shoot tip size, moreover it has possibility to replace meristem culture (Wang and Valkonen, 2009).

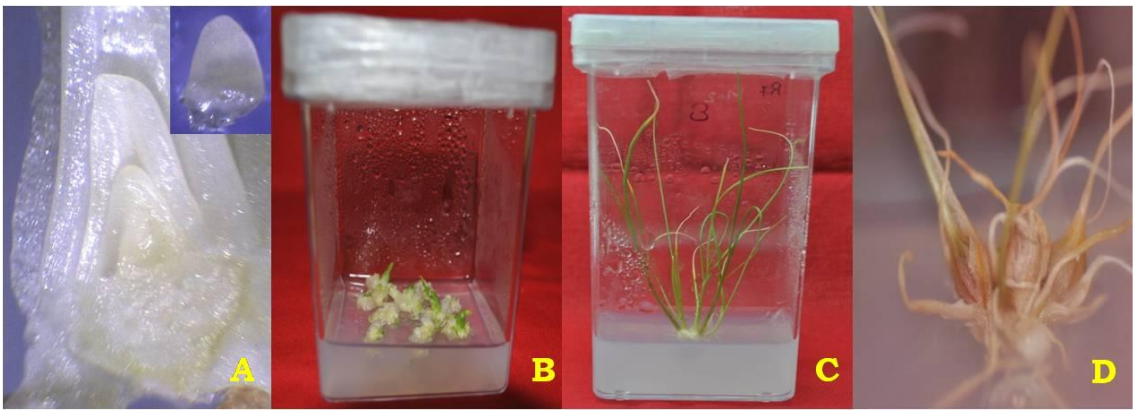
Somatic embryogenesis

Somatic embryogenesis in garlic was first reported in 1977 by Abo–El–Nil, who obtained embryos through differentiation of calli obtained from stem tips. Embryos are generated from basal plates and floral receptacles of garlic plant (Xue *et al.,* 1991; Al–Zahim *et al.,* 1999; Sata *et al.,* 2001). Fereol *et al.,* (2002) produced somatic embryos from calli obtained by culture of root tip which comprised of meristematic tissue. The somatic embryos developed into plants which could be established in greenhouse. Somatic embryogenesis has the advantage that more number of plants can be obtained from calli, but there are chances of genetic fidelity due to plants regenerated through callus. Thus, our need is to produce virus–free plants through direct somatic embryognesis.

Table.1 Research reports on virus–free garlic seed production by meristem culture

Reference	Results
Abo–el–nil (1977)	Garlic plants were established for first time through meristem tissue culture from the variety Extra–Early–White.
Osawa *et al.,* (1981)	Adventitious multiple garlic shoots are obtained by indirect regeneration from meristem tissue through callus formation.
Messiaen *et al.,* (1994)	Numerous adventitious shoot buds are produced from three garlic varieties through meristem tissue culture.
Ucman *et al.,* (1998)	OYDV eliminated in garlic plants of cultivar Ptujksi–spomladanski through thermotherapy and meristem tissue culture by culturing meristems of 0.3–0.6 mm size.
Ebi *et al.,* (2000)	Mite–borne mosaic virus eliminated through meristem tissue culture by isolating meristems of 0.2–0.4 mm from bulbils.
Senula *et al.,* (2000)	87 garlic accessions were made free from LYSV, OYDV, SLV, GCLV and MbFV through meristem tissue culture of 0.3–0.8 mm meristems.
Salomon (2002)	Meristems of up to 0.5 mm used as explants for *in–vitro* tissue culture of garlic resulted in virus–free plants.
Haque *et al.,* (2003)	Garlic plants were regenerated and bulbs induced from shoot and root meristems of the cultivar Bangladesh Local.
Cecilia *et al.,* (2010)	Garlic plant obtained through meristem culture were tested for viruses by DAS–ELISA and ISEM–D, clones free of viruses were obtained and evaluated in field for bulb yield. Virus–free plants yielded 137% higher than virus infected plants.
Hatira *et al.,* (2013)	Hundred per cent virus–free garlic plants were obtained through meristem culture in MS medium containing 0.5 mg/L of 2–isopentenyladenine (2–iP), 0.2 mg/L of NAA and 30 g/L of sucrose as well on MS medium containing 2 mg/L of BA, 0.5 mg/L of indole–3–butyric acid (IBA) with 30 g/L of sucrose.

Figure.1 *In–vitro* meristem culture, A) Meristem with protective miniature leaf scales, B) Shoot differentiation from ≈0.4 mm size of meristem, C) Elongation of differentiated shoot, D) *In–vitro* bulbing of meristem cultured shoots and matured bulb ready for harvest (Source: ICAR-DOGR, Pune, India)



Prospects

The above review shows that virus–free plants have been regenerated from different parts of garlic plants. Among the various plant organs, the root tips containing meristem tissue has the advantage of providing more number of explants, since more number of roots is available per cloves (30 or more). Hence, generally roots developed from bulbs in *in–vitro* are used for plant tissue culture (Robledo–Paz *et al.*, 2000). Use of meristem culture in combination with thermotherapy and chemotherapy increases the efficiency of production of virus–free plants. However, it is valuable to mention that *in–vitro* propagation is sometimes associated with hyper–hydricity or vitrification, which limits the successful establishment of tissue culture plants upon transfer into greenhouse. Hyper–hydric plants are slow in growth and have thick translucent deformed stems with wet leaves (Olmos and Hellin, 1998; Kevers *et al.*, 2004). These disorders are reflected in abnormalities at anatomical and physiological levels. Wu *et al.*, (2009) carried out biochemical and ultra structural analysis of hyperhydric garlic shoots regenerated *in–vitro* and observed that organelles namely mitochondria and chloroplasts were compressed against the cell wall in shoots.

Presently production of virus–free garlic seed production and their multiplication is restricted to laboratory scale. There is need to develop commercial scale protocols for production of virus–free seed cloves planting material. To achieve this, need is to standardize the protocols for multiple shoot production from virus–free plants in a short period and their successful establishment *in–vivo* to produce the bulbs or cloves of normal size to be of use for raising the crop under field conditions. Starting with the virus–free plants, four to five years would be necessary to obtain virus–free elite seeds that can be established in the field.

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