IN VITRO CULTURE TECHNIQUES FOR DISEASE FREE PROPAGULES PRODUCTION IN GARLIC (*ALLIUM SATIVUM*), A SPICY VEGETABLE WITH THERAPEUTIC CHARACTERISTICS

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Abstract. Garlic has been successfully propagated using various micropropagation methods both through direct and indirect organogenesis. *In vitro* micropropagation from the inflorescence, root tip somatic embryogenesis, shoot multiplication in liquid cultures, shoot tip culture, meristem culture, stem-disc dome culture, stem-disc culture has been reported. Therapeutic techniques have been deployed to produce planting material devoid of viruses. This review comprehends various factors affecting morphogenesis and propagule production in garlic.
Keywords: *garlic, micropropagation, somatic embryogenesis, basal disc, microbulblets, virus elimination*

Introduction

Garlic (*Allium sativum* L.) is an ancient domesticated spice crop which belongs to the genus Allium, a most diverse genus in the family Alliaceae with roughly 114 species. It is a diploid (2n =16) plant belonging to monocotyledons and is vegetatively propagated through cloves (Shemesh-Mayer and Kamenetsky-Goldstein, 2021). It is preferably quoted as a medicinal plant because of its therapeutic properties like anti-bacterial, antifungal, antiprotozoal, antiviral, antioxidant, anti-inflammatory, immunomodulatory activity, anti-SARSCoV-2 (Severe Acute Respiratory Syndrome Corona virus 2) and is also used for the culinary purposes. The medicinal and aromatic properties of garlic are due to organosulphur compounds mainly allicin (Mösbauer et al., 2021).

Therapeutic uses of garlic

Since time immemorial garlic has been the part of natural therapeutics as exemplified its usage by Greek physicians Hippocrates and Galen to treat intestinal and extra-intestinal diseases; ancient Japanese and Chinese for headache, flu, and sore throat (Jaber and Al Mossawi, 2007). Garlic acts as digestive system protectant, anti-obesity, neuroprotective, renal protective and used in the preparation of various functional foods and nutraceuticals for treatment of various diseases (Shang et al., 2019). It is used in treating chronic degenerative disease like obesity, diabetics, atherosclerosis, and hypertension (Morales-González et al., 2019). Garlic derived products such as

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phytocompounds, extracts and nano formulations are evaluated for the various stages of cancers in skin, ovary, prostate, breast, gastric, colorectal, oral, liver and pancreas (Mondal et al., 2022).

In garlic, organosulphur compounds and their derivatives are majorly responsible for the exertion of bioactive characteristics, with Allicin (diallyl thiosulphonate) forming a major contributor while diallyl sulphide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E-ajoene, Z-ajoene, S-allyl-cysteine (SAC), and S-allylcysteine sulphoxide (alliin) are the other derivatives of major organosulphur compounds (Rouf et al., 2020).

The phytochemicals in garlic promotes expression of super oxide dismutase (SOD) and catalase antioxidant enzymes and increases the glutathione levels in the cells (Liu et al., 1992; Borlinghaus et al., 2014). Diet supplementation of garlic at 80-4000 mg day^{-1} for 2-24 weeks reduces the malondialdehyde levels and boosts antioxidant capacity (Askari et al., 2021). Similarly, *in vitro* studies reported the antioxidant activity of ajoene, a byproduct of garlic through the activation of nuclear factor 2 (Nrf2) thereby triggering the regulation of chain of events in raising glutathione levels and other genes encoding the cysteine-metabolizing enzymes (Kay et al., 2010). Allicin weakens the response to oxidative stresses and regulates cell apoptosis (Liu et al., 2015). Studies conducted with human endothelial cells showed aged garlic extracts (AGE) increased expression of glutamate cysteine Ligase and heme oxygenase-1 enzymes (Hiramatsu et al., 2016). Oral administration of AGE to male albino rats at dose of 100–200 mg kg−1 has shown gastroprotective effect against the gastric inflammation (Lee et al., 2011).

Antimicrobial activity of garlic extracts has been demonstrated in important bacterial infections with *Bacillus cereus*, *Escherichia coli*, *Klebsiella* spp., *Micrococcus* spp. and *Staphylococcus aureus* (Nakamoto et al., 2020). Similarly, the antifungal properties of garlic could be seen against the fungus, *Penicillium funiculosum* and *Candida albicans* (Li et al., 2016). Consumers demand of garlic has been at all time high during the Covid-19 pandemic due to its therapeutic effect in strengthening immune system by reduced secretion of the cytokine and leptin which are proinflammatory in nature (Donma and Donma, 2020).

Propagation methods and problem associated with the conventional propagation

Garlic has very low reproduction coefficients and is vegetatively propagated through cloves due to its inability to produce viable seeds (Fan et al., 2022). Genetic diversity of garlic is severely limited due to a lack of meiotic recombination and the accumulation of somatic mutations which also aggravate disease transmission over generations resulting in damage to commercial clones through poor seed material (Malik et al., 2020).

Micropropagation in garlic

Garlic has been successfully propagated using various micropropagation methods both through direct and indirect organogenesis. *In vitro* micropropagation from the inflorescence, root tip somatic embryogenesis, shoot multiplication in liquid cultures, shoot tip culture, meristem culture, stem-disc dome culture, stem-disc culture, and somatic embryogenesis has been reported by several authors. Thermotherapy followed by meristem culture techniques has also been used to produce virus free planting materials.

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Direct organogenesis

Direct organogenesis in garlic was established from a variety of explants including root apex, stem-disc, shoot apex, inflorescence, and meristem. The explants and the media composition play a significant impact on the shooting response. The size, quality, genotype of explants and addition of hormones had a significant role in effectiveness of micropropagation (Smith, 2012).

Stem-disc explant

The condensed stem located inside the garlic cloves is referred to as the "basal disc" and it is a modified stem that has leaves above ground. It was reported to be a productive explant for garlic micropropagation. The *in vitro* propagation and bulblet development in garlic cv. Block, Chets, Shaw, #72, and Elephant garlic were reported by Seabrook (1994). The explant basal plate having axillary buds with one or two scales performed better in MS medium containing 2 mg L^{-1} BAP or 2iP and 0.5 μM NAA. Ayabe and Sumi (1998) reported that, basal-disc of the garlic variety "Fukuchi-howaito" which was cold treated (4 °C for eight weeks) prior to inoculation for eight weeks at 4 °C and grown on basal LS media produced 20–30 shoots after three weeks and 90% of the *in vitro* shoots developed bulblets after one month of culture. To eradicate viruses from virus-infected garlic plants, stem-disc dome culture was established for the garlic cultivars Fukuchi-howaito and Hokkaido-zairai (Ayabe and Sumi, 2001).

The Chinese jiaotou *Allium chinense* stem disc culture was initiated in MS media supplemented with 1 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA, which induced a maximum of 17 shoots per explant when subcultured in media containing 1.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA for 8 weeks. For bulblet initiation and development from shoots, media was enriched with 12 % sucrose and 30 °C temperature condition was found to be optimum (Xu et al., 2008).Similarly a direct organogenesis was attempted in Ooty 2 garlic (Meena et al., 2021).The workflow for disease free micropropagules production is represented (*Fig. 1*) and microbulblets successfully produced (*Fig. 2*) from the shoots of basal stem discs (unpublished).

Root tip explant

The production of large number of cloves through micropropagation prefers root tips as an explant. The tip of the root had a meristem region that differentiated into shoots within four weeks period (Haque et al., 1999).The maximum shooting of 95% was seen in root tip explants that were taken from 15 to 18 days old sprouted cloves cultured in solidified agar. The maximum shooting (75%) was observed in MS media added with 10 μM BAP and 1 μM NAA from root tip explant.

In garlic cv. White roppen, protocol for *in vitro* propagation through for cyclic propagation was established using root tips by Haque et al. (1998a). The protocol includes three steps *viz.* shoot induction, shoot proliferation and bulblet induction, which requires MS media supplemented with 1 μM NAA and 10 μM BAP, MS media with 0.5 μM BAP and MS media added with 12% sucrose respectively. Among eight garlic varieties, garlic cv. White roppen responded well for bulblet development through direct organogenesis using root tips as explant. The single clove inoculated in MS media enriched with 10 μM BAP and 1 μM NAA produced 40 root tips with a shooting capacity of 95 %. The bulblets were produced in media containing 12% sucrose using *in vitro* shoots at an average of 7.5 bulblets per explant (Haque et al., 1998b).

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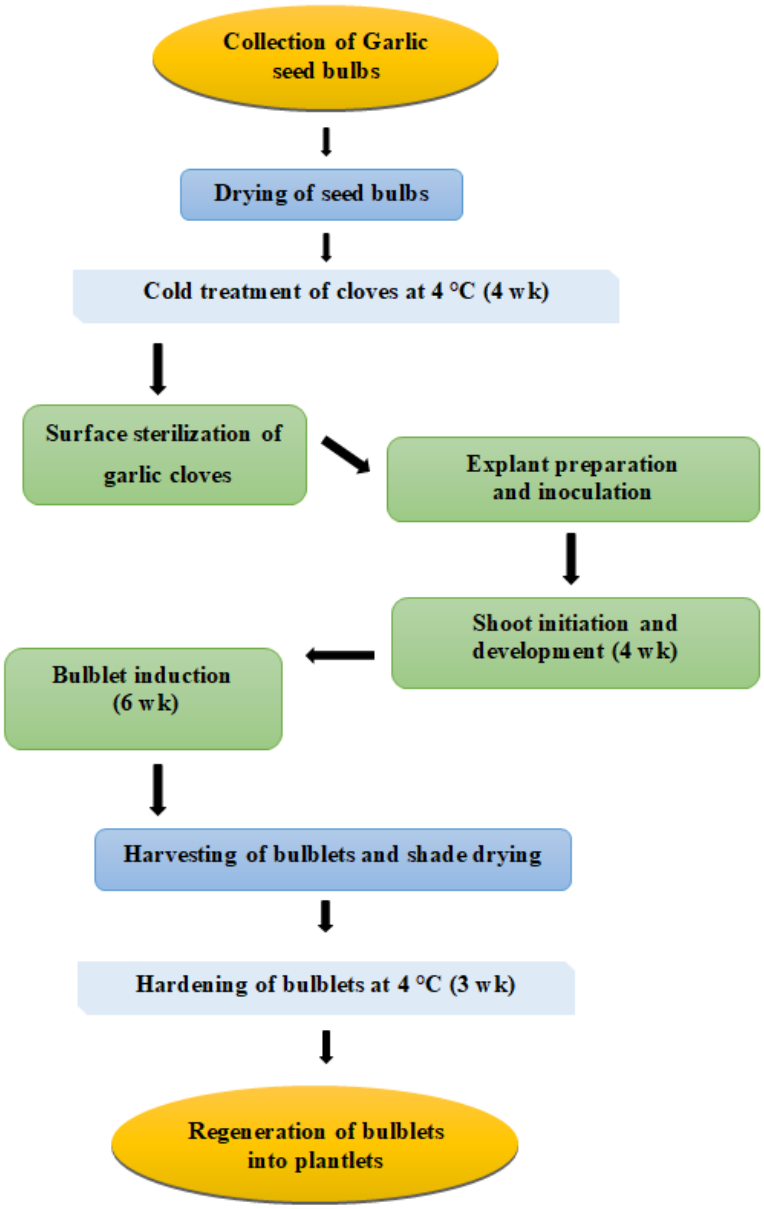

Figure 1. *Workflow of disease-free propagules production in garlic*

Figure 2. *Harvested Garlic bulblets (micropropagules) from in vitro cultures*

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Haque et al. (2003) reported a protocol for regenerating local garlic of Bangladesh using shoots and root meristem as explants. The maximum shooting (95.55%) was observed in hormone free MS media using shoot meristem as explant and bulblets were produced from shoots in MS basal without hormones,12% sucrose supplemented MS media as well as 0.5 µM BAP supplemented MS media. Haque and Hattori (2017) studied the elimination of viruses from Japanese garlic cultivar White roppen and Bangladeshi garlic clones G1, G3, and G14, using root tip culture in MS media containing 10 µM BAP and 1 µM NAA. Clones were found to be free of Leek yellow stripe virus (LYSV) based on RT-PCR (Reverse transcription couple Polymerase Chain Reaction) examination of the shoots confirming the viral-free garlic plant production from infected mother plants. Bulb development in garlic has been reviewed recently and is influenced by several factors like plant population, photoperiod, soil nutrients and structural properties, cold conditioning, temperature, growth regulators, clove weight and genetic responses of plants (Khokhar, 2023).

Shoot tips explant

Nagakubo et al. (1993) established a technique for multiple shoot induction and bulblet generation from shoot tip culture in two late maturing garlic cultivars *viz.*, Furano and Howaito-roppen, and four early maturing cultivars *viz.*, Isshuwase, Isshu-gokuwase, Shanhai and Santo. The modified LS media having 10 µM BAP and 5 µM NAA induced multiple shoots due to increased ratio of KNO_3/NH_4Cl. The early maturing cultivar shoots produced bulblets in basal LS media whereas late maturing cultivars produced bulblets in LS media containing 6% to 12% sucrose after the cold treatment. In garlic and shallots, micropropagation for regeneration of shoot and induction of bulblets were reported by Mohamed-Yasseen et al. (1994) in MS media enriched with BAP or TDZ. The explants were made by making longitudinal incisions in the shoot tip area of cold-treated garlic and shallots by excluding the basal section. According to his theory, multiple shoots were induced due to slicing shoot tip explant. The bulblets were induced from shoots in MS media added with 12 % sucrose and 5 g activated charcoal under long-day photoperiod culture conditions and the bulblets produced were capable of germinating without the acclimatization step.

The Tunceli garlic (*Allium tuncelianum*) shoot and root tip culture was reported by Yanmaz et al. (2010). MS media added with 0.1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA in shoot tip culture produced 1 to 2 shoots per explant and found to be more effective than root tip culture. In addition, bulblet formation from shoots was observed in MS media without any hormones. The development of new varieties of garlic chives (*Allium tuberosum* Rottl. Ex Sprang) is restricted by their apomictic character. High shoot regeneration was seen during micropropagation by using mesocotyl axis obtained from *in vitro*-grown seedlings in MS medium supplemented with 1 mg L^{-1} BAP and 2 mg L^{-1} IBA. The basal media added with 0.5 mg L^{-1} IBA was used for rooting.

For the shoot tip culture and *in vitro* bulb development in the garlic *cv.* Jonas, media supplemented with 1.07 µM IAA generated the greatest response. In response to the addition of ABA, growth was retarded whereas the jasmonic acid addition has stimulated bulbing (Vieira et al., 2014). Meristem and shoot tip explants from Lumbu Hijau, Tawang Mangu, and Lumbu Putih were used to study the response of the different garlic cultivars in MS media supplemented with 2 mg L^{-1} IAA, 2 mg L^{-1} Kin and 0.01 mg L^{-1} GA_3. Higher growth percentage was observed in shoot tip as compared to meristem explant (Karjadi and Gunaeni, 2018; Karjadi and Aswani, 2021).

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Inflorescence explant

Wen et al. (2020) investigated the high frequency of shoot organogenesis of inflorescence explants by optimizing medium, pH and explant size using 14 commercially available garlic cultivars. The average shoot regeneration rate was 97% and the average number of shoots per explant was 23.4. The shoots were regenerated directly without callus formation, which was confirmed by histological observation. Similarly, the inflorescence explant producing systemic virus-free garlic was reported in Baodi garlic 'Liu Ban Hong' by Fan et al. (2017). An average of 11 buds per explant was produced in MS media enriched with 2 mg L^{-1} Kin and 0.1 mg L^{-1} NAA. The coefficient of *in vitro* bulblet propagation was 10.64 per explant. Onion Yellow Dwarf Virus (OYDV), LYSV and Garlic Mosaic Virus (GMV) absence was confirmed by ELISA analysis.

Fan et al. (2022) established *in vitro* Micropropagation of garlic 'Jiangjin No.1' using inflorescence as an explant. The inflorescence explants at the harvest stage were divided into three groups based on the length of the flower and stem to pseudo stem ratio *viz.* A - 1:1.2, B - 0.8:0.9, and C - 0.6:0.7 to optimize the best time to harvest, group two was found to be an optimum time to harvest due to the high efficiency of bulblet induction with a propagation coefficient of average 23 bulbs per explant. *In vitro* produced garlic bulblets were taken up for two-generation cultivation where they found there was no split or blotting in garlic clove in first generation bulbs but in the second generation, multiple cloves were obtained from a single bulb and those seed materials were able to germinate and perform like mother explant in all agronomic characters.

Clove explant

The cloves of garlic local cv. Mukteshwar were used for *in vitro* culture. The cloves were soaked for 24 hr in different treatment solutions for sprouting with combinations of GA_3 and water and transferred to MS media, where they found 85% of sprouting in MS medium with 2 mg L^{-1} GA_3 which were soaked in 100 ppm GA_3 solution. The best shoot multiplication was observed in MS enriched with 0.5 ppm GA_3 + 1.0 ppm Kin +5 ppm BAP (Karn et al., 2022).

The micropropagation of garlic cv. 'Tawangmangubaru', a high-demand spice in Indonesia using clove as explant was studied under different media combinations having NAA (0, 0.5, 1.0, 1.5 ppm) along with coconut water (0, 10, 20%). The shoot multiplication was maximum in 20 % coconut water containing media without NAA which produced about 15.33 shoots per clove whereas 0.5 ppm NAA enhanced the rooting (Mahmudah et al., 2021).

Indirect organogenesis

The first study on garlic micropropagation was done by El-Nil (1977) using cv. Extra Early White. For callus initiation, explants including stem tips, bulb leaf discs and stem segments were used in semi-solid AZ medium supplemented with 10 µM *p*- CPA, 2 µM 2, 4- D and0.5 µM Kin. AZ medium supplemented 1.0 µM Kin and 10 µM IAA for callus subculture and 40 µM potassium nitrate (KNO_3), 18 µM ammonium dihydrogen phosphate ($NH_4H_2PO_4$) along with 10 µM IAA and 10 µM Kin for shoot organogenesis. Differentiated shoots were observed under electron microscopy for virus screening and it revealed the presence of filamentous viral particles. Higher levels of 2, 4-D was found to be detrimental to the callus initiation in *in vitro* garlic studies. Similarly, the media

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combinations of dicot plants were effective in high-frequency callus induction in garlic (Barandiaran et al., 1999).

The production of callus from the leaf disc, root tip and basal disc explants derived from *in vitro* germinated cloves was reported by Haque et al. (2003a). The percentage of callus produced from the strain G-103 was high in MS media enriched with 1 mg L^{-1} BAP and 2 mg L^{-1} 2, 4-D and the media enriched with 1 mg L^{-1} BAP and 2 mg L^{-1} NAA was found to be optimum for high plant regeneration with a survival rate of 30-40%. High shooting from an embryogenic callus was achieved in the MS medium that included 10 mg L^{-1} BAP. The shoots were rooted in basal MS media and after acclimatization, plants were transferred to soil (Khan et al., 2004).

The leaf petioles were used for callus induction by Mehta et al. (2013). The optimum media for producing the callus was reported as MS+0.25 mg L^{-1} 2,4-D+0.5 mg L^{-1} Kin and 0.25 mg L^{-1} 2,4-D+0.5 mg L^{-1} BAP which produced callus with a diameter of 4.6 cm and 3.4 cm, respectively. Metwally et al. (2014) investigated the *in vitro* regeneration of adventitious shoot organogenesis in three garlic cultivars *viz.*, Sids 40, Balady and VFG 180 (3-1), along the wild type. The callus induction was achieved in both shoot and root apices whereas, the 100% callus induction was achieved using root tips in MS media added with 1 mg L^{-1} 2,4-D+5 mg L^{-1} NAA+5 mg L^{-1} BAP. The highest callus frequency was observed in cv. Balady. The B5 medium containing 10 mg L^{-1} Kin and 2 mg L^{-1} IAA had the highest rate of shoot regeneration from callus obtained from root tips. After acclimatization, the plantlets were transplanted to the field. After three vegetative generations, tissue cultured garlic yielded commercial size bulbs.

The meristem culture was used to produce callus from which plantlets were regenerated. The three stages of plant regeneration were optimized, which include callus induction, callus proliferation and shoot regeneration. The 100% callus induction was obtained in B5 medium added with 1 mg L^{-1} BAP and 0.7 mg L^{-1} 2, 4-D and low level of growth hormones used for callus proliferation in MS media with 0.1 mg L^{-1} BAP and 0.5 mg L^{-1} 2, 4-D. The plantlets were grown in B5 media for regeneration (Benke et al., 2018).

The effect on endogenous callus production was studied in relationship with genotypes and explant by Mostafa et al. (2020). MS media enriched with 0.5 mg L^{-1} NAA+0.2 mg L^{-1} BAP + 3 mg L^{-1} 2,4-D was used for testing upper leaf, part lower leaf, part interior and exterior leaves and tips explants responses in four Chinese garlic varieties T_{167}, Da Xing (CK), T_{141} and T_{36}. In all the varieties, shoot tip best responded for callus production. The shortest culture period of 45 days was taken by the variety T_{141} to produce high-weight calli.

Somatic embryogenesis

Successful somatic embryogenesis depends on various factors like genotype, explant and optimized media components. The root tips explant was used for callus and somatic embryo induction, where callus regeneration capacity was found to be 86.10% in MS media enriched with 1 mg L^{-1} 2,4-D but better regeneration of somatic embryos was found when MS media supplemented with 2 mg L^{-1} Kin used. The callus and somatic embryo production took culture periods of 17 and 10.67 days, respectively. Plantlets were developed in MS basal media (Hassan et al., 2014).

Kereša et al. (2021) reported virus-free plant production in two Croatian garlic ecotypes through somatic embryogenesis. Four different media composition were used to culture the basal part of cloves *viz.* MS basal media, MS media+1 mg L^{-1} 2,4-D+0.5 mg

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L^{-1} Kin and modified MS media (2x $MgSO_4$,1/4th of KNO_3 and NH_4NO_3) +0.1 mg L^{-1} 2,4-D for embryogenic callus induction. The conversion of embryogenic callus into the plantlet was maximum in MS+0.1 mg L^{-1} 2,4-D and for rooting, the media composed of MS+0.005 mg L^{-1} 2iP + 0.05 mg L^{-1} NAA. Viral eradication success rates for the regenerated plants from mother plants that were infected with viruses like OYDV, LYSV, and Garlic Common Latent Virus (GCLV) were 20.7%, 30.5%, and 22.9%, respectively was reported. *In vitro* somatic embryogenesis and multiple shoot generation was studied in Garlic *cv.* 'Blanco Criollo' from Cuba. The VIUSID *Agro*® was added at the rate of 2.5 ml L^{-1} in media as a supplement. The best media for callus induction was found to be ½ MS+20 g L^{-1} sucrose+0.75 mg L^{-1} 2,4-D+1 mg L^{-1} Kin. Complete plant formation was observed in MS+20 g L^{-1} sucrose+1 mg L^{-1} thiamine (Guerra et al., 2021).

The first reports of the production of structures known as embryoids in garlic were made using calli extracted from stem ends, bulb leaf discs grown in kinetin, and IAA (Robledo-Paz and Tovor-Soto, 2012). The development of embryos is necessary to provide genetic variety and to enhance various plant characteristics, including stress tolerance (Lämke and Bäurle, 2017). However, callus culture and embryo production require a specific medium composition, obtaining plant embryos *in vitro* is challenging (Guruprasad et al., 2016).

Liquid culture

In garlic cv. Danyang, (Kim et al., 2003a) investigated three types of culture methods *viz.* semi-solid, liquid-immersion and raft cultures for shoot multiplication and bulblet development using root tip explant. *In vitro* shoots generated by root tip cultures were grown in MS medium with 0.5 mg L^{-1} 2iP and 2% sucrose. The highest shoot multiplication was observed in liquid culture as compared to the other two cultures in three weeks. MS medium enriched with 11% sucrose, 10 µM JA and 0.1 mg L^{-1} NAA was found to be optimum for bulblet production. Darkness as treatment (50 µmol m^{-2} s^{-1} for 16 photoperiods) had a positive effect on bulblet induction and development. Kim et al. (2004) compared the bioreactors for the garlic shoot and bulblet multiplication between temporary immersion (ebb and flow) and continuous immersion (with and without a raft) cultures using already optimized media *viz.*MS+2% sucrose for shoot multiplication and liquid MS+11% sucrose + 0.1 mg L^{-1} NAA for bulblet formation. After a three-week culture period, continuous immersion cultures generated 27 shoots from each explant, which were proven to be superior to temporary immersion cultures for shoot proliferation. For bulblet induction, an immersion-type bioreactor with a raft proved optimal condition. The development of bulblets was enhanced by 3 weeks of cold treatment of the cultures at 4 °C. The bulblets were harvested after 9 weeks of culture period and cold treatment was given for 8 weeks at 4 °C before being transferred to pots for germination.

Factors affecting the morphogenesis

Apart from the discussed explant and the media combinations, there are other factors which could influence the micropropagation in garlic. Slicing of the shoot explants increased the number of shoot formation in garlic cloves due to the wounding effect (Mohamed-Yasseen et al., 1994) Low Temperature effects the bulblet formation in Howaito -roppen garlic cultivar (Nagakubo et al., 1993). Effect of endogenous hormone levels has been studied in the callus formation (Mostafa et al., 2020). Growth retardants

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such as CCC, B-9, and ABA have been found to promote bulblet induction and growth (Kim et al., 2003a).

Due to the faster growth rates that come from a high medium to tissue contact, liquid cultures are typically preferred over solid support systems. Cultured tissue grows more quickly and responds more quickly to changes in medium and selection pressure. Several species have found benefits from liquid cultures in terms of increased shoot growth and proliferation.

Hardening and acclimatization of garlic propagules

In tissue culture process, hardening is an important step. Perhaps it is a more challenging and labour-intensive process. When micropropagated plants are relocated from the stress-free aseptic cultures to natural circumstances, they are exposed to the whims of environmental change and are thus exposed to the quirk of both biotic and abiotic influences (Chandra et al., 2010). When plants are transplanted from *in vitro* conditions to greenhouse or field conditions, a portion of plants usually won't survive. By gradually exposing the plants to the microenvironment, the plants can be helped to successfully adapt to the exterior environment. The plants undergo physiological changes as they adapt to their new habitat, altering the anomalies to help them survive in the field (Pospóšilová et al., 1999).

Mohamed-Yasseen et al. (1994) raised plantlets from the *in vitro* bulblets of garlic and shallot using commercial potting mix for planting. Kim et al. (2003a) used 1:1 ratio of peat moss and perlite potting mix for planting *in vitro* bulblets. Vermiculite was used for the hardening of the regenerated plants. After acclimatization plants were grown using a soil mixture containing clay, sand, and organic matter in the ratio of 1:1:1. Microbiotization with plant growth promoting rhizobacteria (PGPR) has been successfully reported in many crops including garlic. Inoculation of mycorrhiza was reported to enhance the growth of mother-plant and the size of the bulbs irrespective of the genotype evaluated (Lubraco et al., 2000).

Virus elimination methods in garlic

Many commercial varieties of garlic are prone to yield reduction and degeneration due to accumulation of virus due to clonal propagation nature of this crop. This has major implications in garlic breeding, since propagation through bulblets would result in increased viral load in the subsequent generations (Scotton et al., 2013). The viruses that affect garlic are commonly referred to as Garlic Viral Complex (GVC) and are transmitted to the subsequent generation through the bulbs (Manjunathagowda et al., 2021). The viruses include Carlaviruses (aphid transmitted), Potyviruses and Allexiviruses (mites transmitted) (Cafrune et al., 2006). Onion yellow dwarf virus (OYDV) causes 39% to 60% reduction in bulblet weight and Leek yellow stripe virus (LYSV) infection causes 17% to 54% weight reduction in bulblets. Coinfections with these viruses cause furthermore reduction in the bulb yield (Lot et al., 1998).

The application of meristem culture in combination with thermotherapy and chemotherapy can increase the frequency of virus elimination; meristem/shoot tip culture and thermotherapy have been successfully used to eliminate garlic viruses; alternative methods like chemotherapy, cryotherapy, and root meristem culture have also been used to eliminate virus-infected Alliums. Meristem culture is routinely used for eliminating plant viruses because of its high genetic stability, growth potential, and cell division rate.

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Meristem tip culture

Meristem culture using garlic cultivars Bhima Purple and Bhima Omkar were studied for virus-free planting material production. MS media fortified with 0.1 mg L^{-1} NAA and 1 mg L^{-1} KIN produced the maximum shoots in both the cultivars. Microbulbil production was optimum in liquid MS medium supplemented with 6 % sucrose and 1 mg L^{-1} KIN. Two cycle production was required for producing normal sized garlic bulbs. 70% of the obtained plants were found to be free of viruses (Murkute and Gawande 2018).

Thermotherapy

Virus elimination through thermotherapy and meristem culture in garlic was reported by Robert et al. (1998) with 90-100 % elimination of OYDV. Meristem explants reported to have high multiplication rate in media supplemented with 1 µM IAA and 1 µM BAP for shoot initiation and 5 µM jasmonic acid and 5 µM 2iP for shoot multiplication. Thermotherapy was found to have interference in the shoot multiplication process, but it was found to be efficient for OYDV elimination up to 90-100 % as analysed by Enzyme Linked Immuno-Sorbant Assay (ELISA). Bulbs were induced after 10 weeks from the shoots due to the addition of jasmonic acid.

Ramírez-Malagón et al. (2006) reported irregular presence of potyvirus in all parts of the garlic plants since some of the parts were affected and some were free of potyvirus. Two commercial garlic cv. Taiwan and chileno were subjected for thermotherapy (32 °C for a week, 36 °C for 2 weeks and 38 °C for 3 weeks), chemotherapy (205 µM Ribavirin) and meristematic culture. *In vitro* culture after thermotherapy resulted in 63% and 70.9% of Taiwan and Chileno virus-free explants, chemotherapy showed only 27.0-34.8% virus elimination and meristem culture yielded 64% of potyvirus-free explants in both cultivars.

Meristem culture combined with thermotherapy eliminated the Garlic Viral Complex (GVC) in garlic cultivar, Bhima purple. Thermotherapy of the infected garlic bulbs at 37 °C for 90 days followed by the meristem culture eliminated the viruses such as Garlic Common Latent Virus (GCLV), OYDV and Allexiviruses (Manjunathagowda et al., 2021).

The heat tolerance of individual plant species and varieties can vary widely, and it also varies depending on how effectively specific viruses are eliminated from different plants through heat therapy. Drastic reduction in the survival rate of explants with increasing temperature during thermotherapy will be the major disadvantages. Hence the temperature must be optimized for each genotype.

Solar or hot air treatment

Meristem culture and shoot tip culture were compared for the virus-free plant production in *Allium sativum* and *Allium tuncelianum* using MS media with different combination and concentrations of plant growth regulators, MS medium containing 0.2 mg L^{-1} NAA+0.5 mg L^{-1} 2iP and MS media supplemented with 0.5 mg L^{-1} IBA+2 mg L^{-1} BAP. *In vitro* shoots obtained from the meristem culture were free of OYDV and LYSV as analysed by the real-time PCR assay but the regenerated plants from shoot tips showed the presence of viruses (Taşkın et al., 2013).

Solar heat treatment for 5, 10 or 15 d or hot air treatment at 37° C, 40° C or 42° C for 7, 14 or 21, respectively were reported in commercial garlic cv. 'G-1'. Increased treatment duration increased the virus-free plant recovery rate, but it also reduced the regeneration. Meristem from the solar-heat treated and hot-air treated cloves regenerated after 90 d and

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45 d respectively. Solar treatment for 15 d showed 17-18% regeneration with full elimination of GCLV, Shallot Latent Virus (SLV) and OYDV. Hot air treatment at 42 °C for 21 d eliminated all the viruses (GCLV, SLV and OYDV) in 29% of the regenerated plants (Pramesh and Baranwal, 2015).

Chemotherapy

Chemotherapy uses chemicals for the elimination of plant viruses by treating the explants prior to *in vitro* culture. In garlic, the effect of chemotherapy for elimination of Garlic common Latent Virus (GCLV) was compared with the meristem culture by Kudělková et al. (2014). Ribavirin concentration of 25 or 50 mg L^{-1} was used along with meristem culture. 25 mg L^{-1} of Ribavirin showed 100 % virus elimination in garlic genotypes N9A, Anton and Tristan. In case of the genotype Mako, the garlic routed through meristem culture alone resulted in no virus elimination.

Cryotherapy

Low temperature treatments of the explants have been proven to eliminate plant viruses in several crop plants. Cryotherapy to eliminate virus complex such as OYDV, LYSV and GCLV in garlic cv. Jonas was reported by Vieira et al. (2015). Cell structure analysis after cryotherapy and thermotherapy showed that the first layers of meristem dome were free of OYDV. The combined effect of cryotherapy and thermotherapy also increased the survival rate of shoot tips and OYDV, LYSV and GCLV were 90, 100 and 80% eliminated in regenerated plants.

Electrotherapy

Production of OYDV free garlic plants from infected plants using electrotherapy, chemotherapy and meristem isolation along with *in vitro* culture was studied (Soliman et al., 2012). Electrotherapy treatment using current, 15 mA/10 min combined with chemotherapy treatment of 20 mg L^{-1} Virazole responded the best for virus elimination. Among the regenerated plants 85 % of them were viral-free for OYDV.

The success of the electroculture technique mainly depends on the following parameters: dormancy time and essential nutrients. Dormancy is the period when plants adapt to environmental conditions to survive drastic changes. On the other hand, essential nutrients are needed for plant metabolism.

Conclusion

Plant tissue culture has evolved into an important technique for rapid micropropagation of valuable crops and has practical role in crop improvement through supply of quality seed material in larger scale with uniform size and free from pest and diseases. Quality of micropropagules can be ensured by clonal fidelity analysis of the somaclonal variation exhibited during *in vitro* culture as well virus free nature through indexing procedures prior to supply to the farmers. Automated micropropagation using temporary immersion bioreactor systems can serve as a cost-effective option which can provide optimal environment for microprogation of garlic in plant tissue cultures. This will be useful for provision of virus free plant propagules to the garlic farmers which is a major constraint to them, in terms of genetic uniformity, availability and a costly seed input among various factors of cultivation.

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APPENDIX

Table 1. *Micropropagation studies in Garlic (Allium sp.)*

S. No.	Explants	Regeneration pathway	Media combination	References
1.	Stem tips, bulb leaf discs, Stem segments	Indirect organogenesis	AZ+10 µM *p*- CPA+2 µM 2,4- D+ 0.5 µM KIN	El-Nil (1977)
2.	Basal plate with twin scale	Direct organogenesis	MS+0.5 µM NAA+2 mg L^{-1} BAP	Seabrook (1994)
3.	Stem-disc	Direct organogenesis	Basal LS	Ayabe and Sumi (1998)
4.	Root tips	Indirect organogenesis	MS+4.6 µM KIN+4.5 µM 2,4- D	Robledo-Paz et al., (2000)
5.	Root tip	Direct organogenesis	MS+1 µM NAA+ 10 µM BAP	Haque et al.,(1998a)
6.	Stem-disc dome	Direct organogenesis	Basal LS	Ayabe and Sumi (2001)
7.	Shoot meristem	Direct organogenesis	Basal MS	Haque et al.,(2003c)
8.	Root tip	Direct organogenesis	MS+ 0.8 % mannitol	Haque et al.,(2003b)
9.	Root tip	Direct organogenesis-Liquid culture	MS+2 % sucrose+0.5 mg L^{-1} 2iP.11	Kim et al.,(2003a)
10.	Root tip, leaf, basal disc	Indirect organogenesis	MS+5 mg L^{-1} KIN+1.5 mg L^{-1} 2,4-D	Haque et al.,(2003a)
11.	Leaf	Indirect organogenesis	MS+1.0 mg L^{-1} 2,4-D	Kim et al.,(2003b)
12.	Root tip	Direct organogenesis-Immersion bioreactor	MS+2 % sucrose	Kim et al.,(2004)
13.	Root tip	Indirect organogenesis	MS+5 mg L^{-1} KIN+1.5 mg L^{-1} 2,4-D	Khan et al.,(2004)
14.	Stem-disc	Direct organogenesis	MS+0.5 mg L^{-1} NAA+1 mg L^{-1} BAP	Xu et al.,(2008)
15.	Shoot tip	Direct organogenesis	MS+0.1 mg L^{-1} IAA+0.1 mg L^{-1} BAP	Yanmaz et al., (2010)
16.	Node	Direct organogenesis	MS+1.0 mg L^{-1} KIN	Yanmaz et al., (2010)
17.	Root tip	Indirect organogenesis	MS+1 mg L^{-1} 2,4-D+ 5 mg L^{-1} BAP+ 5 mg L^{-1} NAA	Metwally et al.,(2014)
18.	Shoot tip	Direct organogenesis	MS+1.07 µM IAA	Vieira et al.,(2014)
19.	Shoot meristem	Direct organogenesis	MS+0.1 mg L^{-1} NAA+1 mg L^{-1} KIN	Murkute and Gawande (2018)
20.	Root meristem	Indirect organogenesis	B5+0.7 mg L^{-1} 2, 4-D+1 mg L^{-1} BAP	Benke et al.,(2018)
21.	Inflorescence	Direct organogenesis	MS+0.1 mg L^{-1} NAA+2 mg L^{-1} KIN	Fan et al.,(2017)
22.	Shoot tip	Indirect organogenesis	MS+0.5 mg L^{-1} NAA+3 mg L^{-1}, 2,4-D + 0.2 mg L^{-1}BAP	Mostafa et al.,(2020)
23.	Basal disc	Somatic embryogenesis	MS+0.1 mg L^{-1} 2,4-D	Kereša et al.,(2021)
24.	Root tip	Somatic embryogenesis	MS+1 mg L^{-1} of 2,4-D	Hassan et al.,(2014)
25.	Shoot tip	Direct organogenesis	MS+2 mg L^{-1} IAA+2 mg L^{-1} KIN+ 0.01 mg L^{-1} GA_3	Karjadi and Gunaeni, (2018)

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