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Effect of nanoparticle and plant growth regulators on callus induction and sterilization of different explants under *in vitro* culture of broccoli (*Brassica oleracea* var. *italica*)

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Abstract
An *in vitro* efficient complete protocol has been developed for plant regeneration through callus induction in the medicinal plant Broccoli (*Brassica oleracea* var. *italica*) using leaf as ex-plant. Tissue culture is a new approach for production of vigorous and disease free plants with many advantages over conventional methods. Broccoli (*Brassica oleracea* var. *italica*) is a valuable source of antioxidants and secondary metabolites. This study is a first extensive report about the effect of different serialization protocols and plant growth regulators (PGRs), and nanoparticles *viz*. Chitosan (50nm) on different explants disinfection efficiency, callus induction and accumulation in callus cultures of Broccoli. In addition, different sterilization agents were evaluated for disinfection of leaf explants, nodal explant and seed explant. The results revealed that out of 12 treatments on surface sterilization of nodal segment explants, the best treatment was T_{10} consisting of 1.0% (w/v) bavistin for 10 minutes followed by 0.50% (v/v) sodium hypochlorite for 5 minutes duration and 0.25% mercuric chloride (HgCl2) for 1 minute. Similarly for seed explants that treatment T_{11} comprising of 1% (w/v) bavistin for 10 minutes duration followed by 0.50% (v/v) sodium hypochlorite of 15 minutes and mercuric chloride 1% for 1.50 minutes gave 85.51% uncontaminated cultures in case of seed segment explants, which is the highest as compared to all other treatments. The results presented for leaf explants showed that treatment T_7 comprising of 1% (w/v) bavistin for 5 minutes duration followed by 0.50% (v/v) sodium hypochlorite of 3 minutes and mercuric chloride 0.1% for 0.30 minutes gave 88.75% uncontaminated cultures in case of leaf segment explants, which is the highest as compared to all other treatments. Sterilization protocols adversely influenced the explant viability and callus induction and growth. On culture media (cl7) supplemented with 1.5 (mg/l) 2,4-D and 0.75 (mg/l) Chitosan showed the maximum callus induction frequency (CIF) (90.27%),: The results indicated the positive effect of low dosage of nano elicitors on callus induction and explant viability. Furthermore, the callus induction is tightly dependent to the presence of auxin and cytokinin, which stimulate both cell division and cell elongation.

Keywords: Broccoli, indole-3-butyric acid, 1-naphthalene acetic acid, 2,4-d dichlorophenoxyacetic acid, mercuric chloride, sodium hypochlorite, chitosan

1. Introduction
Broccoli (*Brassica oleracea var. italica*) is a member of the Brassicaceae family. Brassicas (Cruciferous crops) are of great economic importance around the world (Francisco *et al*., 2017). Among them broccoli is the most studied and consumed vegetable of the family due to its richness in iron, calcium, minerals and vitamins A, C, E, and K the crop contains 3.3% of protein content and also substantial amount of riboflavin, niacin, and thiamine and also contain high concentration of carotenoids. Broccoli shares cancer fighting and immune boosting properties with other cruciferous vegetables such as cauliflower, Brussels sprouts and cabbage. Plant tissue cultures are the core of plant biology, which is important for conservation, mass propagation, genetic manipulation, bio-active compound production and plant improvement, It is an excellent way to increase the secondary metabolites Plant tissue culture forms an integral part of any plant biotechnology activity. It offers an alternative to conventional vegetative propagation *In vitro* fungal and bacterial contaminations are also one of the most important limiting factors, particularly in woody plants. In order to overcome fungal and bacterial contaminations in the disinfection stage, different surface sterilizing agents including sodium and calcium hypochlorite, ethanol, mercuric chloride, silver nitrate, antibiotics, and fungicide were used in laboratories worldwide. For rape seeds, a method involving soaking in water, sterilization with alcohol and NaClO, and multiple washes with sterile water has been

developed (Fu, Shu, *et al*., 2019) [2]. Additionally, a plant tissue culture explant sterilization apparatus has been designed, which allows for easy sterilization of explants while minimizing loss and controlling sterilization time (De, Ying *et al*., 2017) [3]. Nevertheless, the efficiency of these treatments is low in woody plants, or some of them are very toxic to plant tissues. Genotypic specificity for regeneration is very high, development of suitable regeneration protocol for each genotype is necessary. (Narasimhulu and Chopra) developed protocols for callus induction and shoot regeneration in Brassica carinata using cotyledonary and stem explants. They found that MS medium supplemented with BA and NAA favored callus induction, and combinations of BA and IAA or BA alone regenerated shoots with a high frequency. Seran *et al*. (2007) [4] successfully produced embryogenic callus from leaf explants of tea (*Camellia sinensis*) using MS media with BAP and NAA, and observed the development of collenchyma cells, parenchyma cells, and somatic embryos. Al-Tai and Mohammed, *et al*., (2022) [6] initiated callus from lavender seedling leaves using MS solid medium supplemented with NAA and BA, and observed the development of somatic embryos that later grew into intact lavender plants. (Eizenga and Dahleen (1990) [7] evaluated the regeneration response and meiotic and isozyme changes in regenerants of tall fescue (*Festuca arundinacea*) and found that genotype was the most important variable for callus production and plant regeneration. Silver nanoparticles have been used as an effective surface sterilization agent in plant tissue culture (Pınar, Nartop. (2018) [8]. They have been shown to have no adverse effects on seed germination and *in vitro* plantlet growth. D., Mahendran, *et al*., 2019) [9]. Silver nitrate (AgNO3) and silver nanoparticles (Ag NPs) have been widely used in plant tissue culture to control microbial contaminants and promote callus induction, organogenesis, somatic embryogenesis, and secondary metabolite production. (Sunita, *et al*., 2015) [10]. Overall, silver nanoparticles have proven to be a valuable tool in the sterilization process and promotion of plant tissue culture. The application of nanoparticles has shown promise in facilitating the sterilization process, reducing contamination, and improving the overall success rate of explant culture. Moreover, plant growth regulators have played a pivotal role in promoting callus formation and subsequent organogenesis, which is crucial for the development of healthy plantlets for further applications in breeding and genetic studies.

2. Materials and Methods

2.1. Explant preparation and sterilization

Keeping in consideration the accessibility and population of plant material available, plant material for present study was collected from the research field of division of vegetable sciences and floriculture Skuast Jammu (320-17' to 370-50' N, 720-40' to 80-30' E, 266.00m/872.70ft.) above sea level (m a.s.l.). Wild explants were washed under running tap water followed by washing with detergent solution “Labolene”1% treated with 2-3 drops of surfactant Tween-20 for 20 minutes. The explants were sterilized using different protocols as described below:-

All nanoparticles were purchased from sigma Aldrich CA, U.S.A) company having different particle size Chitosan solution were prepared by dissolving them in few drops of ethanol or acetic acid before making up the final volume with distilled water and, Silver nitrate were prepared by dissolving them in distilled water as they are soluble in water and were properly refrigerated until further use

Sterilization protocol: The effects of different concentrations of Bavistein, NaOCl, mercuric chloride and pH adjustment of NaOCl on the sterilization and growth of explants

2.2. For nodal Explant

The nodal explants were dipped in different doses of bavistin (w/v) ranging from 0.1 to 1.0% for 10 minutes, followed by sodium hypochlorite (v/v) ranging from 0.1 to 1.0% for 5 minutes and mercuric chloride for 0.1 to 0.50%(w/v) for 1 minute on surface sterilization of nodal explant as shown in the Table.1. After that, nodal were rinsed with sterile distilled water (SDW) for three times and then sliced into small fragments (0.5 cm squares), and cultured on MS medium supplemented with different concentrations of auxins [α-naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mg/L concentrations] and nanoparticle Chitosan (50nm) 0.75 mg/l

Table 1: Different treatment doses of bavistin (w/v) for 5 minutes sodium hypochlorite (v/v) for 3 minutes and mercuric chloride for 30 seconds. for surface sterilization of nodal explants.

Treatment (T)	Treatment doses of bavistin (%) for 10 minutes	Treatment doses of sodium hypochlorite (%) for 5 minutes	Treatment of HgCl2 (%) for 1 minute
T_1	0.1	0.1	0.1
T_2	0.25	0.1	0.1
T_3	0.1	0.25	0.25
T_4	0.25	0.1	0.25
T_5	0.25	0.25	0.50
T_6	0.75	0.1	0.25
T_7	1.0	0.5	0.1
T_8	0.75	1.0	0.50
T_9	0.1	0.75	0.25
T_{10}	1	0.5	0.25
T_{11}	1	.75	.50
T_{12}	.50	1	.50

2.3. For leaf explants

The leaf explants were dipped in different doses of bavistin (w/v) ranging from 0.1- 1.0% for 5 minutes, followed by sodium hypochlorite (v/v) ranging from 0.1-1.0% for 3 minutes, and 0.1% to 0.50% mercuric chloride (HgCl2) for 30 seconds on surface sterilization, was observed as shown in the Table 2. After that, leaf explants were rinsed with sterile distilled water (SDW) for three times and then sliced into

small fragments (0.5 cm squares), and cultured on MS medium supplemented with different concentrations of auxins [α-naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mg/L concentrations] and nanoparticle Chitosan (50nm) 0.75 mg/l

Table 2: Different treatment doses of bavistin (w/v) for 5 minutes sodium hypochlorite (v/v) for 3 minutes and mercuric chloride for 30 seconds for surface sterilization of seed explant.

Treatment (T)	Treatment doses of bavistin (%) for 5 minutes	Treatment doses of sodium hypochlorite (%) for 3 minutes	Treatment of HgCl2 (%) for.30 minute
T_1	0.1	0.1	0.1
T_2	0.1	0.5	0.1
T_3	0.5	0.25	0.25
T_4	0.25	0.1	0.25
T_5	1.0	0.25	0.50
T_6	0.75	0.1	0.25
T_7	1.0	0.5	0.1
T_8	0.1	1.0	0.1
T_9	0.5	0.75	0.25
T_{10}	.75	0.5	0.25

2.4. For seed explant

The seed explants were dipped in different doses of bavistin (w/v) ranging from 0.1 to 1.0% for 10 minutes, followed by sodium hypochlorite (v/v) ranging from 0.1 to 1.0% for 15 minutes and mercuric chloride for 0.1 to 0.50%(w/v) for 1.5 minute on surface sterilization of seed explant as shown in the Table 3. After that, leaf explants were rinsed with sterile distilled water (SDW) for three times and then sliced into small fragments (0.5 cm squares), and cultured on MS medium supplemented with different concentrations of auxins [α-naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mg/L concentrations] and nanoparticle Chitosan (50nm) 0.75 mg/l.

Table 3: Different treatment doses of bavistin (w/v) for 10 minutes sodium hypochlorite (v/v) for 15 minutes and mercuric chloride for 1.5 minute for surface sterilization of seed explant.

Treatment (T)	Treatment doses of bavistin (%) for 10 minutes	Treatment doses of sodium hypochlorite (%) for 15 minutes	Treatment of HgCl2 (%) for 1.5 minute
T_1	0.1	0.1	0.1
T_2	0.25	0.5	0.1
T_3	0.5	0.75	0.25
T_4	0.25	0.25	0.75
T_5	0.1	0.75	0.50
T_6	0.75	0.1	0.25
T_7	.25	1.0	0.1
T_8	0.1	1.0	0.1
T_9	0.1	0.75	0.25
T_{10}	1.0	0.5	0.25
T_{11}	1	0.5	1
T_{12}	0.5	0.5	1
T_{13}	1	.25	1

2.5. Medium preparation and cultures incubation condition

MS medium were supplemented with 3% sucrose and solidified using 0.8% plant agar. The pH of the medium was adjusted to 5.7–5.8 before autoclaving at 121 °C for 20 min. Cultures were maintained in a growth chamber at 24 ± 1 °C and 16-h photoperiod (cool Wight florescent light; 55 µmol/m^2s) and sub-cultured at monthly intervals. The percentage of callus induction, bacterial and fungal contaminations, viable and browning explants was recorded 4 weeks after culture. The callus fresh weight (mg/single explant) was measured 3 months after culture.

2.6. Callus Induction

Chitosan nanoparticles were purchased from sigma Aldrich CA, U.S.A) company having (40-50nm) particle size. Surface sterilized leaf segments were inoculated on solid MS medium containing sucrose (3% w/v), agar (0.8% w/v) and various concentrations of growth regulators for *in vitro* culture establishment. For callus induction, MS media was supplemented with different concentrations of 2,4-D and Chitosan nanoparticles (30-50nm), chitosan were prepared by dissolving them in few drops of ethanol or acetic acid before making up the final volume with distilled water (Table 4). The cultures were kept in darkness for seven days to induce callus before being transferred to a 16/8 hour photoperiod for three weeks. Each treatment had six repetitions (Petri dishes), with four to five (1.0-1.5cm) leaf explants in each Petri dish, with slight modifications. After four weeks of inoculation, following observations were recorded: percentage of explants producing callus [callus induction frequency (CIF)], callus induction response, as well as the type of callus.

Table 4: Effect of growth regulators with Chitosan added in MS medium for callus induction from leaf explants

S.no.	Growth regulator (mg/l)	Nanoparticle (mg/l)
	2,4-D	Chitosan (40nm)
CI1	0.0	0.0
CI2	0.1	0.25
CI3	0.25	0.50
CI4	0.50	0.50
CI5	0.75	0.50
CI6	1.0	0.75
CI7	1.50	0.75
CI8	2.00	0.75
CI9	2.50	1.0
CI10	2.50	1.0

2.7. Statistical analysis

Disinfection experiments conducted in a factorial (disinfection and PGRs as factors) arrangement based on a completely randomized design with three replicates with 12 explants per replication. Measurements carried out in a completely randomized design (CRD) with three replications. Experimental data subjected to analysis of variance (ANOVA) ($p < 0.05$) and using statistical software OPSTAT

(http:// 14.139.232.166/opstat/ index.asp). The critical difference was calculated at 5% probability level.

3. Results and Discussions

3.1. For nodal explant

While for nodal segment explants, Table 1 reveals that out of 12 treatments on surface sterilization of nodal segment explants, the best treatment was T_{10} consisting of 1.0% (w/v) bavistin for 10 minutes followed by 0.50% (v/v) sodium hypochlorite for 5 minutes duration and 0.25% mercuric chloride (HgCl2) for 1 minute. This treatment (T_{10}) resulted in 80.1% uncontaminated cultures, which was significantly different in compare to other treatments. Our results were similar with Dodake *et al.* (2020) [11] who found that *Momordica dioica* and *M. cochinchinensis*, both belonging to the Cucurbitaceae family, are tuberous rooted and can be sterilized using surface sterilization techniques. Hashim *et al.* (2021) [12] investigated the sterilization of *Clinacanthus nutans* nodal explants and found that mercuric chloride (HgCl2) at a concentration of 0.2% for 1 hour resulted in the highest percentage of survival and viability. Bello *et al.* (2018) studied the surface sterilization of Solanecio biafrae nodal explants and proposed a protocol using 70% ethanol for 20 seconds followed by 10% calcium hypochlorite for 15 minutes. Sonali, Das. (2020) [14]. found that sterilization with 0.1% HgCl2 for 10 minutes followed by 1% NaOCl for 2 minutes reduced contamination in Tagetes erecta nodal explants. Daud *et al.* (2012) [15] investigated different sterilization regimes for *Aquilaria malaccensis* and found that pre-sterilization with 0.2% Benomyl followed by surface sterilization with HgCl2 yielded the best results for leaf and nodal explants.

For seed explant

Another sterilization protocol result in table 2 showed that treatment T_{11} comprising of 1% (w/v) bavistin for 10 minutes duration followed by 0.50% (v/v) sodium hypochlorite of 15 minutes and mercuric chloride 1% for 1.50 minutes gave 85.51% uncontaminated cultures in case of seed segment explants, which is the highest as compared to all other treatments. Our result was similar with the study of Khanam and Chandra (2017) [85] found that a combination of mercuric chloride (0.1% for 5-10 min) and ethanol (70% for 6-8 min) showed the best sterilization efficiency for plant explants. Gunawardhana *et al.* (2020) [76] determined that the optimal sterilization protocol for *Piper nigrum* L. was specific to the explant type, with the third leaf and apical bud showing the least contamination and browning. They recommended protocols containing 70% ethanol (30s), 0.1% HgCl2 (5 min), sterile distilled water with activated charcoal (1 gL-1; 25 min), and 20% sodium hypochlorite (NaOCl) (15 min) with 70% ethanol (1 min) for the third leaf, and 0.1% HgCl2 (10 min) and 70% ethanol (1 min), and 10% NaOCl (15 min) with 70% ethanol (1 min) for apical buds. Wegayehu, *et al*., (2015) found that pre-sterilization using 0.2% Benomyl for 15 minutes followed by surface sterilization using mercury chloride (HgCl2) yielded the best results for leaf and nodal explants of *Aquilaria malaccensis*

3.2. For leaf explant

The results presented in Table 3 shows that treatment T_7 comprising of 1% (w/v) bavistin for 5 minutes duration followed by 0.50% (v/v) sodium hypochlorite of 3 minutes and mercuric chloride 0.1% for 0.30 minutes gave 88.75% uncontaminated cultures in case of leaf segment explants, which is the highest as compared to all other treatments. Explants contamination resulted from sterilant treatments at lower concentrations, but treatments at higher doses resulted in browning, loss of juvenility, and eventually the explants did not responded properly to media and eventually died. Our finding from the present investigation showed that the leaf and nodal segments are more susceptible to fungal contamination, thus increasing the bavistin concentration was very much effective; however, increasing the sodium hypochlorite concentration makes the plants less sensitive in the culture. The reason for this might be that the NaOCl has a high oxidation capability, which is extremely powerful against bacteria, fungi, and viruses present on the surface of the explants and infest the deep tissues of the explants. The reaction between amino acids and NaOCl yields the aldehyde, NH4Cl, and CO_2 (Yildiz *et al*., 2012) [77]. As a result, direct contact of the tissue with NaOCl during sterilizing process damage the tissue's mild to heavy but it depends on the concentration and application time of sodium hypochlorite. Sundararajan, S., *et al*. (2015) [16] discusses a modified surface sterilization protocol for *Brassica oleracea* var. botrytis (cauliflower) explants using sodium hypochlorite. It explores the effects of different concentrations and exposure times on successful sterilization. In another study revealed that Leaf explant sterilization is a crucial step in broccoli tissue culture (Iyyakkannu *et al*., 2021) [1]. Different methods have been proposed to improve the efficiency of sterilization. One approach is the use of fumigation-based methods, such as turmeric and benzoin resin-based fumigation, which have shown promising results in achieving contamination-free plant tissue culture (Zeng, *et al*., 2019) [68]. Another method involves rinsing the explants in tap water for a specific duration, followed by sterilization using 75% ethanol and a sterilizing agent (Zhang, *et al*., 2017) [67]. Additionally, a plant tissue culture explant sterilization apparatus has been developed, which allows for easy sterilization of explants while avoiding loss and ensuring controllable sterilization time(De, Ying., *et al*., 2017) [3] These methods aim to address the challenge of contamination in tissue culture and improve the success rate of *in vitro* regeneration of broccoli explants.

3.3. Callus induction in Broccoli using leaf as explants

After ten days of culture incubation, callus initiation was observed at the cut ends of leaf explants. The data shown in Table 3.5 reveals that growth regulators had a considerable impact on percent callus induction, there is no callus development observed in control culture medium in which no growth regulators added. The treatment CI7, which fortified with 1.5 mg/l 2,4-D in combination with 0.75 mg/l Chitosan produced the highest callus induction rate (90.47%) when compared with other treatments. The callus was compact, green, and quick respond to growth. The callus formed by lower concentrations of 2,4-D, on the other hand, was friable and slow to expand. There was a boost in percent callus induction when the concentration of 2,4-D was raised up to 1.50 mg/l, followed by a decrease in percent callus induction and a slowing of callus growth as the concentration of 2,4-D was increased further (Plate 1).our result was similar with El-Bakry, A. A., *et al*. (2020) [86] that investigates the impact of chitosan on callus induction and plantlet regeneration in date palm tissue cultures. Chitosan is found to enhance callus

formation and the subsequent regeneration of plantlets, suggesting its potential for improving tissue culture protocols. Similarly, Sharma, S., Niranjan, A., *et al* (2020) [78] focuses on the use of chitosan to enhance shoot regeneration and somatic embryogenesis in Capsicum chinense (Naga King Chili). Chitosan treatment is reported to significantly improve callus formation and subsequent plant regeneration. Jorapur *et al*. (2018) [79] obtained 100 percent callusing with MS supplemented with 1.5 mg/l 2,4-D and 0.5 mg/l NAA. Thakur and Kanwar (2017) [87] found highest callus induction when MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l NAA. Callus induction in broccoli using leaf explants has been studied in several papers. Kumar *et al*. 2018) [80] investigated the effect of different treatment concentrations on callus induction and shoot multiplication in gerbera plants. They found that the combination of 2,4-D at 2.00 mg/l and BAP at 2.00 mg/l resulted in the maximum time for callus induction (Rajesh, *et al*., 2022) [66]. Yeasmin *et al*. developed an efficient *in vitro* regeneration protocol for strawberry plants using leaf explants. They found that the highest percentage of callus induction was obtained on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IBA (Sahida, *et al*., 2022) [65]. (Zhou *et al*., 2022) [81] studied the effects of different concentrations of compound sodium nitrophenolate (CSN), silver nitrate, and diethyl aminoethyl hexanoate (DA-6) on callus induction in *Jatropha curcas*. They found that adding 8 mg/L CSN or 10 mg/L DA-6 increased the callus induction rate of leaf explants. (Yazıcılar and Chang 2022) [82] investigated callus and embryogenic callus induction in alfalfa. They found that callus formation was detected at a rate of 74% in different cultivars, and the embryogenic callus differentiation varied among cultivars. (Wang *et al*., 2021) [83] explored alternative explants for maize regeneration and found that root tips and young leaves from seedlings could generate embryogenic calli. The rate of primary callus induction from root tips was 97.2%. Kamran, M., & Parveen, A. (2019) [84]. Investigates the effect of chitosan on callus induction and regeneration in rice (Oryza sativa). Chitosan treatment is found to enhance callus production, suggesting its potential as a valuable supplement in rice tissue culture protocols. Examines the influence of chitosan on callus formation in cultivated calla plants (*Zantedeschia* spp.). Chitosan is reported to positively affect callus induction, potentially facilitating tissue culture applications in ornamental plants.

4. Conclusion

In the pursuit of achieving optimal surface sterilization and subsequent successful *in vitro* culture of nodal segment, seed segment, and leaf segment explants in our study, a series of carefully designed treatments were evaluated. These treatments spanned a range of concentrations and durations for bavistin, sodium hypochlorite, and mercuric chloride. Through rigorous experimentation, we have unveiled a wealth of insights into the delicate balance between achieving sterilization and preserving explant vitality. It is noteworthy that while these sterilization protocols demonstrated their efficacy in mitigating contamination, they did exert an influence on explant viability and callus induction and growth. This serves as a reminder of the intricate trade-offs inherent in tissue culture techniques, where the pursuit of sterility must be harmonized with the preservation of cellular vitality and regenerative potential.

In summation, our research has not only shed light on the intricate nuances of surface sterilization for different explant types but also underscored the pivotal role of culture media supplementation in promoting desirable outcomes. These findings provide valuable insights for advancing the field of plant tissue culture, with implications spanning from plant propagation to genetic studies and beyond. As we continue to refine our understanding of these techniques, the possibilities for innovative applications in agriculture and biotechnology remain boundless.

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