International Journal of Horticultural Science and Technology
Vol. 1, No. 2; December 2014, pp 131-140

The Application of Essential Oils and Silver Nanoparticles for Sterilization of Bermudagrass Explants in *in vitro* Culture

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(Received: 10 March 2014, Accepted: 30 June 2014)

Abstract
An important part of plant *in vitro* techniques is the sterilization of explants and the maintenance of aseptic conditions. Ideally, sterilizing materials should be effective on a vast range of microorganisms at low density. Nowadays, the use of compounds such as essential oils (EOs) and nanoparticles is applicable in microbiology studies. The main objective of this experiment was to study the substitution probability of silver nanoparticles (SNPs), thymol and carvacrol as novel sterilization agents in the tissue culture of *Cynodon dactylon*. Explants were sterilized with 70% ethanol for 2 min, and then 30% Clorox for 15 min. Sterilization complementary treatments (SNPs, thymol and carvacrol) were applied at different concentrations (100 and 200 mg l^{-1}) with exposure times of 30, 60 and 120 min. According to the results, infection of bermudagrass explants was controlled successfully by SNPs, thymol and carvacrol. Examination of various concentrations in different exposure times showed that 200 mg L^{-1} SNPs in combination with 100 mg L^{-1} thymol in 60 min inhibited microbial growth. Thymol and carvacrol were more effective than SNPs in controlling bacteria and fungi contaminations. Finally, these novel agents could be used as an alternative to common chemical treatments for elimination and control of microbial population explants in *in vitro* conditions.

Keywords: antimicrobial, SNPs, thymol, carvacrol, tissue culture.

Abbreviation: **SNPs**, silver nanoparticles; **EOs**, essential oils; **2,4-D**,2,4-dichlorophenoxyacetic acid; **NaOH**, sodium hydroxide; **h**, hour; **CRD**, completely randomized design; **cv**, cultivar.

Introduction

An important part of plant *in vitro* culture is the sterilization of explants and the maintenance of aseptic conditions. Different microorganisms rapidly grow in an *in vitro* culture medium because of hydrocarbon sources (such as sucrose), and ultimately infect the plant cells. Plant organs and tissues are sterilized with different disinfecting solutions. Bacteria and fungi are the two most troublesome contaminants that are observed in *in vitro* cultures. To eliminate microorganism contamination during *in vitro* propagation, the most commonly used solutions are calcium hypochlorite, sodium hypochlorite, hydrogen peroxide, ethylene alcohol, silver nitrate, mercury chloride and antibiotics (Torres, 1989). Despite the success of this method in many cases, the resistance of bacteria and fungi to common bactericides and fungicides in a period of time after contamination is a limiting factor in the

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sterilization of plant and media materials. Also, some antimicrobial chemicals have phytotoxic signs, like reduction of explants' growth rate or genetic mutations. Therefore, there is much interest in new substitutions which are safe and cost-effective antimicrobial materials (Sondi and Salopek-Sondi, 2004).

Silver-based compounds such as silver nitrate have long been recognized as highly toxic to microorganisms, and show strong antibacterial activity on broad strains of bacteria (Sondi and Salopek-Sondi, 2004). Nanoparticles have unique chemical, physical and optical features, due to the fact that they have higher surface volume in comparison with bulk materials that are made of ions. Thus, it is expected that silver nanoparticles (SNPs) would be more efficient in catalytic reactivity and antimicrobial activity than silver microparticles, and present a reasonable alternative for development of new bactericidal materials (Sondi and Salopek-Sondi, 2004; Kim *et al*., 2007; Navarro *et al*., 2008). Some reports have shown that antimicrobial components in the form of nanoparticles could be used as bactericidal materials in the plant tissue culture. Abdi (2008) observed an optimum result for explant disinfection by using 120 mg l^{-1} of nano-silver solution after surface sterilization of valerian tissue culture, without any deleterious effect on explants. Rostami and Shahsavar (2009) reported that adding nano-silver with concentration of 4 mg L^{-1} to MS media was effective to control olive explants' contaminations, without any harmful effects on growth. Gharati *et al*. (2010) studied the effect of different concentrations of nano-silver solution on Persian walnut (*Juglans regie* L.) contaminations in the *in vitro* culture condition. They found that the optimal condition for decreasing the indigenous bacterial contamination on walnut segment nodes was 75 ppm. Safavi *et al*. (2011) evaluated the potential for nano-silver to remove contaminants of tobacco explants in MS medium, and their results showed that nano-silver had good potential for removing the bacterial contaminants in tobacco tissue culture procedures. Adding nano-silver at 50 mg L^{-1} concentration to media was fully effective in controlling the microorganism infection. Gholamhoseinpour Anvari *et al*. (2012) evaluated suitable disinfecting agents for control of bacterial contaminations of peach × almond hybrids in the tissue culture conditions. Results indicated that the addition of 10 to 20 mg m^{-3} nano-silver directly to the culture media was effective for control of bacterial growth in the culture media. In another experiment, Fakhrfeshani *et al*. (2012) studied the antimicrobial activity of a different nano-silver solution on gerbera tissue culture. They reported that 200 mg L^{-1} nano-silver solution had successfully controlled bacterial and fungal contamination without having any deleterious effect on the regeneration of plantlets.

On the other hand, the use of natural compounds such as essential oils (EOs) could be a promising strategy for antimicrobial purposes. EOs are volatile terpenes which are naturally produced by aromatic plants. Several studies have investigated how plant-derived essential oils such as carvacrol, thymol, eugenol, limonene and borneol may be effective alternatives to overcome microbial resistance. They are also organic natural substances which suggest both public health safety and environmental friendliness. Carvacrol is the major component (50-80%) of essential oils in some medicinal plants such as thyme, oregano, savory and zataria (Macheboeuf *et al*., 2008; Martinez-Romero *et al*., 2007; Sharififar *et al*., 2007). Carvacrol has a phenolic structure and its antimicrobial activity has been recently proved against bacteria (Olasupo *et al*., 2007; Botelho *et al*., 2007), fungi and yeast (Martinez-Romero *et al*., 2007; Yahyazadeh *et al*., 2008). Thymol, one of the major compositions of thyme oil, has been cited

as an effective antifungal and antibacterial with potential applications for control of plant diseases (Solgi *et al*., 2009; Braga *et al*., 2008; Yahyazadeh *et al*., 2008; Olasupo *et al*., 2007; Botelho *et al*., 2007). However, there are no available reports about application of SNPs-linked EOs for sterilization of explants in *in vitro* culture.

Bermudagrass (*Cynodon dactylon*) is still considered a warm-season plant with low maintenance requirements, and is a good adaptation of turf grass for tissue culture. Combinations of hypochlorite and ethanol are usually applied for disinfecting bermudagrass vegetative tissue (Lu *et al*., 2006). Our preliminary experiment showed that the rate of contamination was high when explants were derived from stolons. Therefore, the main objective of this experiment was to study the potential substitution of SNPs, thymol and carvacrol as novel disinfecting agents in tissue culture of bermudagrass with common chemical treatments.

Materials and Methods

Plant materials and sterilization treatments

Bermudagrass (*Cynodon dactylon* cv. 'Teefgreen') stolons in the vegetative stage were collected from planted pots in a controlled greenhouse. Old leaves were removed and the stolons were cut into pieces, each having one node. Nodes were rinsed with running tap water for 20 minutes, sterilized with 70% ethanol for 2 min, and then with 30% Clorox (Golrang bleach) for 15 min, followed by three rinses with distilled water in sterile laminar air flow (Taghizadeh *et al*., 2012). The sterilization complementary treatments SNPs (Nanocid Co., Iran.), thymol (Sigma Co.) and carvacrol (Merck Co.) were applied at 100 and 200 mg L^{-1} concentrations in different soaking times (30, 60 and 120 min). Treatments were compared with distilled water (DW) (without any disinfecting agent) and the common method (CM) (ethanol plus Clorox). After that, explants were rinsed five times with sterilized distilled water.

Media and condition of culture

After sterilization treatments, the nodal segments were cultured on MS basal medium supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar and 2 mg L^{-1} 2,4-D (Taghizadeh *et al*., 2012). The pH of media was adjusted to 5.8 with 0.2 N NaOH prior to autoclaving. Then, media was autoclaved at 121ºC for 20 min. The described media were poured into 10cm Petri dishes (approximately 25 ml per dish). Cultures were kept at 24 ± 2ºC for a 16/8h light/dark photoperiod for callus induction. The percentage of bacteria and fungi contaminations, callus production and necrosis were recorded from three days after culture. Estimation of colonies' size was performed on the 10^{th} day. In this regard, the disinfecting agent, agent concentration, soaking times and their interactions were studied.

Experimental design and data analysis

Each treatment had four replications with five explants in each replication. A completely randomized design (CRD) with factorial arrangements was used for the experiment. Data were analysed using the ANOVA procedure of the SAS statistical software. Duncan's multiple range tests (DMRT) were performed for mean comparisons at the 5% probability level.

Results

The effects of the disinfecting agent on sterilization of bermudagrass nodes are shown in Table 1. The effects of different disinfecting agents were significant on appearance, infection of contamination, callus production and necrosis sign ($P \leq 0.01$). In distilled water (DW), the fungi and bacteria infected the explants at rates of 100% and 40%, respectively, and this parameter was significantly lower in treated explants (which were, on average,

20% fungi and 19.5% bacteria infected). Also, there were distinct differences between various agents compared with distilled water on colony appearance and diameter of fungi, bacterial contamination and explant total infection. The data indicated that fungi colonies appeared only after three days, while the bacterial contamination was observed four days after culture in the common method. Colony appearance was delayed by 5.6-7.7 and 4.8-7.8 days for fungi and bacteria contaminations, respectively, when different disinfecting agents were applied. Also, the infection percentages were 26.7% and 20% for fungi and bacteria contaminations, respectively, when using ethanol plus hypochlorite as a common method. Therefore, the common method is not useful for controlling explant contamination of bermudagrass in *in vitro* culture. Among treatments, SNPs sharply reduced total infection (22.2%) and delayed infection of colony appearance (7.7-7.8 days) compared with distilled water and the common method. Also, colony size decreased when SNPs were applied for sterilization (7.6 mm and 1.2 mm for fungi and bacteria, respectively) compared with distilled water and the common method. The lowest infection percentages of explants (12.2% and 10% for fungi and bacteria, respectively) was recorded using SNPs. Therefore, SNP treatment efficiently inhibited growth of both fungi and bacteria contaminants in bermudagrass explants. Application of thymol and carvacrol treatments was significant for reducing fungi and bacteria growth as well as total infection in comparison with distilled water. Results showed that colony appearance of fungi was delayed (6.2 days) by using carvacrol and colony appearance of bacteria was delayed (5.6 days) by using thymol treatment. Treatments of SNPs, thymol and carvacrol had no effect on callus formation in comparison with distilled water and common method during *in vitro* culture of bermudagrass ($P \leq 0.01$). Additionally, explant necrosis percentage was not significant among treatments.

Table 1. Effects of different disinfecting agents (SNPs, thymol and carvacrol) on disinfection of bermudagrass nodal explants in *in vitro* culture.

Treatment	Appearance (days)		Infection(%)		Diameter (mm)		Total infection (%)	Necrosis (%)	Callus (%)
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria			
Nanosilver	7.7^{a}	7.8^{a}	12.2^{d}	10^{c}	7.6^{c}	1.2^{c}	22.2^{c}	17.8^{a}	14.4^{a}
Thymol	5.6^{c}	6.5^{b}	23.3^{cb}	20.6^{cb}	20.4^{b}	3.8^{cb}	42.8^{b}	20.3^{a}	6.1^{ab}
Carvacrol	6.2^{b}	4.8	18.1^{cd}	28.6^{ab}	19.42^{b}	6.8^{a}	46.1^{b}	17.2^{a}	13.3^{a}
CM	3.0^{d}	4.0^{c}	26.7b	20^{cb}	18.3^{b}	3.2^{cb}	46.7^{b}	13.3^{a}	13.3^{a}
DW	3.0^{d}	4.0^{c}	100^{a}	40^{a}	31.8^{a}	5^{ab}	100^{a}	-†	-†

Mean values followed by different letters are significantly different.
† All explants were eliminated by bacteria and fungi contaminations during this experiment.

Effects of different concentrations of disinfecting agents were significant for all parameters except percentage of necrosis of explants ($P \leq 0.01$) (Table 2). The data indicated that by increasing concentrations (200 mg L^{-1}), infection percentage (27.7%), colony appearance (6.7 days) and colony size (11.7 mm) of fungi reduced, while percentage of bacterial infection (28.3%), colony size (4.8 mm) and callus initiation were increased (14.4%).

Table 2. Effects of different concentrations of disinfecting agents (100 and 200 mg L^{-1}) on sterilization of bermudagrass nodal explants in *in vitro* culture.

Treatment Concentration (mg l^{-1})	Appearance (days)		Infection (%)		Diameter (mm)		Total infection (%)	Callus (%)
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria		
100	3.5^{b}	6.5^{a}	44.4^{a}	19.1^{b}	27.3^{a}	3.2^{b}	55.6^{a}	4.4^{b}
200	6.7^{a}	4.8^{b}	27.7^{b}	28.3a	11.7^{b}	4.8^{a}	47.6^{b}	14.4^{a}

Mean values followed by different letters are significantly different.

Interaction effects between disinfecting agents and different concentrations were significant on contamination appearance and infection percentage factors ($P \leq 0.01$). Among treatments, the highest percentage of total infection (55.6%) was obtained by using 100 mg l^{-1} carvacrol percentage (Table 3). Treatment with 200 mg l^{-1} SNP decreased total infection (11.1%) especially for fungi contamination (0%). The lowest total infection (11.1%) was observed with 200 mg L^{-1} SNP treatment, with no fungi colony (0%) in this treatment. The highest percentage of bacterial contamination of explants was detected using 100 mg L^{-1} thymol (37.8%) and 200 mg L^{-1} thymol (36.7%).

Table 3. Interaction effects of disinfecting agent and concentration on sterilization of bermudagrass nodal explants in *in vitro* culture.

Treatment	Appearance (days)		Infection (%)		Diameter (mm)		Total infection (%)
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	
Nanosilver							
100 (mg L^{-1})	5.3c	8.2a	24.4c	8.9c	15.2b	1.1c	33.3c
200 (mg L^{-1})	10a	7.3ab	0d	11.1c	0c	1.2c	11.1d
Thymol							
100 (mg L^{-1})	3.8d	8.8a	37.8b	4.4c	35a	0.7c	42.2cb
200 (mg L^{-1})	8.2b	4.1cd	8.9d	36.7ab	5.8c	6.8ab	43.3cb
Carvacrol							
100 (mg L^{-1})	3.2d	5.6c	33.3bc	22.2bc	36.3a	5.9ab	55.6b
200 (mg L^{-1})	9.2ab	3d	2.7d	33.9ab	2.6c	7.7a	36.7cb
CM	3d	5.7bc	26.7bc	20bc	18.3b	3.2bc	46.7cb
DW	3d	4cd	100a	40a	31.8a	5abc	100a

Mean values followed by different letters are significantly different.

Interaction effects of disinfecting agents and exposure time on some sterilization factors, including bacteria appearance, fungi infection, fungi diameter and total infection of explants, were significant ($P \leq 0.01$). In relation to the interaction between disinfecting agent and exposure time (Table 4), the highest total infection percentage (57.5%) was obtained by using carvacrol for 30 min, and the lowest total infection percentage (14.2%) was recorded by using SNP for 120 min. Increasing the period of exposure time had a positive effect on controlling contamination. Increasing the exposure time of SNPs and thymol treatments delayed bacterial colony appearance (7.5-9 days) compared with distilled water and common method (4-5.7 days). Also, increasing the period of exposure with SNPs treatment (60-120 min) led to appropriate control of total infection (14.2-16.7%) compared to CM and DD treatments (46.7- 100%).

Table 4. Interaction effects of disinfecting agents and exposure time on sterilization of bermudagrass nodal explants in *in vitro* culture.

Treatment	Appearance (days)	Infection (%)	Diameter (mm)	Total infection (%)
	Bacteria	Fungi	Fungi	
Nanosilver				
30	5.7c	16.7bc	12.3 b	35.8bcd
60	8.8a	10c	5.3b	16.7d
120	9a	10c	5.2b	14.2d
Thymol				
30	5.2cb	36.7b	26.2ab	56.7b
60	6.8ab	23.3cb	22ab	38.3bcd
120	7.5ab	10c	15ab	33.3bcd
Carvacrol				
30	3.5c	23.3cb	30.6a	57.5b
60	4c	20.8cb	14.2ab	50.8bc
120	5.3cb	10c	13.5ab	30cd
CM	5.7cb	26.7cb	18.3ab	46.7bc
DW	4c	100a	31.8a	100a

Mean values followed by different letters are significantly different.

Discussion

Many factors influence the effectiveness of chemical disinfectants and antiseptics. Factors such as the kinds of microorganisms, the concentration and nature of the disinfectant used, and the length of treatment should be considered. Ideally, the disinfectant must be effective against a wide range of microbial agents at low concentrations (Kumar, 2001). In the *in vitro* propagation of bermudagrass (*Cynodon dactylon* L.), the application of SNPs and EOs as two antimicrobial agents prevents successful contamination of Bermudagrass nodal explants. When SNPs was used for decontamination, it inhibited fungi and bacterial growth. No previous study has investigated the effects of SNPs and EQs on decontamination explants simultaneously in *in vitro* culture. However, there are some *in vitro* culture studies which confirm the disinfecting properties of SNPs on explants (Abdi *et al*., 2008; Rostami and Shahsavar, 2009; Gharati *et al*., 2010; Safavi *et al*., 2011; Gholamhoseinpour Anvari *et al*., 2012; Fakhrfeshani *et al*., 2012).

The antimicrobial activity mechanism of silver that silver nanoparticles release has been considered among a wide range of microorganisms, including its alteration of cell membrane structure and functions. It has been proposed that silver ions strongly substitute the sulphur in thiol groups of vital enzyme and proteins, inactivate them, disturb metabolism and lead to the death of bacteria cells. Moreover, silver ions can interact with DNA and prevent bacterial replication ability. When these ions are prepared in the nanoparticle form, they are expected to show high antimicrobial activity because of their larger specific surface compared with bulk silver metal (Maneerung *et al*., 2008; Morone *et al*., 2005). Based on these results, inhibition of contamination depends on the concentration and exposure time of the agent on explants. The high concentration and prolonged exposure time of applied SNP were effective in controlling contamination. It seems that these agents could have an excellent biocide effect, especially given that these agents are more effective in reducing fungi growth factors including diameter, appearance and colony

development. Similar results were reported by Abdi *et al*. (2008) and Rostami and Shahsavar (2009). They found the best results for disinfection in valerian and olive came from using 100 mg L^{-1} SNP. Also, Abdi *et al.* (2008) have shown that increasing the concentration and period of treatment caused better control of disinfected valerian nodal explants. As an explanation for this inconsistency, it could be pointed out that they applied very low concentrations (25-100 mg L^{-1}) in valerian and olive, whereas we used high concentrations of SNPs (100 and 200 mg L^{-1}). In most treatments, microbial growth decreased dramatically when agent concentration and exposure time were increased in the sterilization solutions. Similar results have been reported in tulip bulb explants by using $HgCl_2$ and benomile (Taghizadeh, 2004). Since the treatments had no negative effect on the callus induction percentage of nodal explants, the necrosis signs did not show any significant effect in different treatments in comparison with control. In this way, Abdi *et al.* (2008) described any significant differences in the measured character of valerian explants when SNPs solution was used for sterilizing *in vitro* culture. On the other hand, the results indicated that thymol and carvacrol inhibited contamination growth; thymol specifically controlled bacterial contamination and carvacrol specifically controlled fungi contamination. According to Loziene *et al*. (2007), EOs compounds such as thymol are antiseptic, while carvacrol possesses antifungal properties. There are no available reports on the effects of EOs for decontamination of explants *in vitro* conditions, but there are some studies about the mode of action of thymol and carvacrol on microbial infections (Loziene *et al*., 2007; Yahyazadeh *et al*., 2007; Martinez-Romero *et al*., 2007; Feng and Zheng, 2007; Knowles *et al*., 2004; Braga *et al*., 2008; Bounatirou *et al*., 2007). Production of EOs by plants is believed to act predominantly as a defence mechanism against pathogens and pests. Indeed, EOs has been shown to possess antimicrobial and antifungal properties (Feng and Zheng, 2007).

It appears that thymol and carvacrol act on microbial cells and their components due to phenolic compound and the fact they have a hydroxyl group. An important characteristic of thymol and carvacrol is hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, binding to membrane proteins and releasing lipopolysaccharides, and thus disturbing the structures and rendering them more permeable (Braga *et al*., 2008; Juven *et al*., 1994; Sikkema *et al*., 1994). Finally, extensive loss of cell contents or the exit of critical molecules and ions will lead to microbial death (Macheboeuf *et al*., 2008; Lambert *et al*., 2001; Skandamis *et al*., 2001; Carson *et al*., 2002). This study demonstrates the effect of EOs treatments on microbial elimination of explants in the *in vitro* culture. Disinfection of nodal explant by all EOs treatments sufficiently eliminated fungal rather than bacterial contamination. Similarly, Martinez-Romero (2007) showed that carvacrol (0.05-1 ml L^{-1}) was highly effective in reducing the growth of *Botrytis cinerea* in table grapes. The mechanism action of carvacrol against fungi is not fully known. However, activity may be linked to solubility in water and the ability to mass through the fungi cell membrane's collapse and the deterioration of the conidia and hyphae (Knobloch *et al*., 1988; Zambonelli *et al*., 2004). Moreover, thymol is one of the major components of thyme oil, has a phenolic structure and is credited with a series of pharmacological properties, including antimicrobial and antifungal effects (Braga, 2008). As noted by Knobloch *et al*. (1988), the variation in the fungicidal action of essential oil components seems to be dependent on their water-solubility and lipophilic properties

(Knobloch *et al*., 1989). A substance may inhibit the growth of fungi either temporarily (fungi static) or permanently (fungicidal). The antifungal activation of EOs was dependent on the concentration (Feng and Zheng, 2006). According to these results, EOs proved to have more fungicidal action at high concentration (200 mg L^{-1}) than bactericidal action. As an explanation for this case, it can be noted that the fungi hyphae have chitin-based cell walls. Thus, EOs penetration is more complex than the simple bacterial cell wall. In general, EOs was less effective in microbial inhibition for nodal explants in comparison with control, which is probably due to the low concentration in which it was applied.

Conclusions

Infection of bermudagrass nodal explant (fungi and bacteria) was controlled successfully by various SNPs, especially with 200 mg L^{-1} SNPs for 60 min. Examination of various concentrations and different exposure times for thymol and carvacrol showed that this compound relativity inhibited growth contamination. However, for an acceptable influence on contamination control (fungi and bacteria), it is better that 200 mg L^{-1} SNPs plus 100 mg L^{-1} thymol for 60 min be used, in order to prevent any adverse effects on growth of bermudagrass explants in tissue culture. We should emphasize that the current study is the first survey on the understanding of thymol and carvacrol's decontamination properties against microbial infection in *in vitro* conditions. So, we propose further research in order to test high concentrations and other EOs for decontamination of explants in *in vitro* culture. Finally, these novel agents, especially SNPs, could be used as an alternative to common chemical procedures for the elimination and control of microbial populations of explants in the *in vitro* condition.

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