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Research Article

STANDARDIZATION OF IN VITRO STERILIZATION AND CALLUS INDUCTION PROTOCOL FOR LEAF EXPLANTS OF ANCHOTE : *COCCINIA ABYSSINICA*

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ABSTRACT

The present study was conducted to develop efficient sterilization and callus induction protocols of Anchote leaf explants. Anchote (Coccinia abyssinica) roots were collected from West Ethiopia transported to Kenya where they were grown in the net greenhouse in Juja. Leaves explants were harvested from the seedlings and used for both sterilization and callus induction protocols. Young leaf explants were selected from healthy plants and subjected sterilization using 5 and 10 % commercial bleach of(NaOCl (3.85%) /(JIK®))at 5 ,10 and 15 minutes intervals. Maximum clean survival explants were obtained (82.5±0.5) at 5%NaOC with 10Minutes) . Best maximum callus induction(80±2) was achieved from the combination of 5µm(BAP+2,4-D).
Keywords: Anchote (Cocciniaabyysinica), Leaf explants, callus, sterilization, BAP, 2,4 -D and NAA.

INTRODUCTION

Anchote[Coccinia abyssinica (Lam.) Cogn.] is annual trailing vine belonging to family of cuccurbitacceae and grown principally for its root as food. It is one of the potential major roots and tuber cultivated plants produced on nearly 300ha of the land on in wollega and produced for food, cultural, social and economical crop for the communities produce it. Anchote can be propagated both vegetative and from seeds. The genus Coccinia is made up of 30 species of which eight are reported(Mengesha et al.,2012) .

Of the major tuberous vegetables such as sweet potato, Oromo potato, and others cultivated in the area, in west Oromia, Ethiopia with its annual yield of 25,000 tones (Anonymous, 2011).Anchote is endemic to the Western parts of Ethiopia (Getahun, 1973), mainly in the Western region of Ethiopia highlands in Eastern Wollega, Western Wollega, Kelam Wollega, and Mattu. It is a valuable food source and according to local farmers, it helps in fast mending of broken/ fracture bones and displaced joints, as it contains high calcium, and proteins than other common and wide spread root and tuber crops (Endashaw, 2007). Traditionally, it is also believed that, Anchote makes lactating mothers healthier and stronger (Abera , 1995). Dawit and Estifanos reported that the juice prepared from tubers of Anchote has saponin as an active substance and is used to treat Gonorrhea, Tuberculosis, and Tumor Cancer.

Tissue culture techniques are used extensively to grow many different plants for commercial and research purposes (Hussain et al, 2012). New plants are grown from small pieces of plant tissue in a nutrient medium under sterile conditions. When conditions are suitable, plants can be induced to rapidly produce new shoots, which can be subdivided to produce more plants. The addition of suitable hormones can then induce root growth, and the plants can then be placed in soil and grown in the normal manner (Rand, 2001). Plant Cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future (Kumar and Kumar, 1996). Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations(Sidorov .2013).

Plant tissues inherently have various bacteria and fungi on their surfaces. It is important that the explants be devoid of any surface contaminants prior to tissue culture since contaminants can grow in the culture medium, rendering the culture non sterile. In addition, they compete with the plant tissue for nutrition, thus depriving the plant tissue of nutrients. Bacteria and especially fungi can rapidly overtake plant tis Sterilization is the process of makingexplants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival (CPRI, 1992). The surface sterility chosen for an experiment typically depend on the type of explants and also plant species (Rezadost et al.,2013).Explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field-grown tissues. The time of sterilization is dependent on the type of tissue; for example, leaf tissue will require a shorter sterilization time than will seeds with a tough seed coa (Funguomali et al.,2013, Sharma and Nautiya ,2009).

Wetting agents such as Tween added to the sterilant can improve surface contact with the tissue. Although surface contamination can be eliminated by sterilization, it is very difficult to remove contaminants that are present inside the explants that may show up at a later stage in culture. This internal contamination can be controlled to a certain extent by frequent transfer to fresh medium or by the use of a low concentration of antibiotics in the medium. Overexposing tissues to decontaminating chemicals can also kill tissues, so there is a balancing act between sterilizing explants and killing the explants themselves(Qin et al., 2012 and Olewet al., 2014).

Callus is an unorganized mass of cells that develops when cells are wounded and is very useful for many in vitro cultures. Callus is developed when the explant is cultured on media conducive to undifferentiated cell production—usually the absence of organogenesis (organ production) can lead to callus proliferation (Mungole et a.l,2011) .Stated another way, callus production often leads to organogenesis, but once callus begins to form organs, callus production is halted. Auxins and cytokinins both aid in the formation of most callus cells(Ali et a.l, 2007).Callus can be continuously proliferated using plant growth hormones or then directed to form organs or somatic embryos .

MATERIALS AND METHODS

Plant materials

Anchote (Coccinia abyssinica) roots were collected from West Ethiopia transported to Kenya where they were grown in the net greenhouse in Juja. Leaves were harvested from the seedlings and used for both sterilization and callus induction protocols.

Fig. 1 Anchote (*Coccinia abyssinica*) grown in net greenhouse

Media preparation

The Murashige and Skoog (1962) media was used for all the experiments. Media was prepared by dissolving the organic and inorganic components in distilled water. The solution was stirred until dissolved and made up to final volume. The

media pH was adjusted between 5.7 and 5.8 by using either 1N HCl or 1N NaOH before the gelling agent was added. Media was then heated on a hot plate with continuous stirring using a magnetic stirrer until agar is dissolved and media dispensed in the culture vessels. The culture vessels were capped with lids and placed in trays and autoclaved. Autoclave was set at a temperature of 121oC and a pressure of 1.1kg/cm2 for 20 minutes. All media was autoclaved within 12 hours of preparation and when possible freshly autoclaved media was used. However, when it was not possible to use the media immediately it was stored in a refrigerator at 4ºC for no longer than two weeks before use.

Plant growth regulators

Plant growth regulators were weighed and stocks were clearly labeled and stored in the refrigerator at 4oC.

Aseptic techniques

The process of sterilization and dissection of plant materials was carried out under sterile conditions in lamina flow cabinet. The cabinet was switched on and swabbed down with 70% ethanol using cotton wool or sterile towel and kept running for about 15 minutes before the work in the cabinet starts. All the plant materials were dissected on the sterile papers. The lamina flow cabinet was frequently swabbed down with 70% alcohol. Hands were sprayed with 70% ethanol at suitable intervals while working for protracted periods in front of the cabinets. Personal hygienic precautions were observed by wearing a clean lab coat and gloves while working in the lamina flow cabinet.

Dissecting tools

All tools were placed in an aluminum foil and sterilized in an autoclave. During their use in the cabinet, tools were dipped in 70% ethanol followed by heat sterilization in steribead sterilizer maintained at 250oC. In between operations, the tools were frequently sterilized by dipping them in ethanol and in the steribead sterilizer for 30 seconds.

Washing of glassware and vessels

All glassware and vessels were washed in hot water to which few drops of liquid detergent had been added. The glassware were then rinsed in cold water three times followed by a final rinse in distilled water with a few drops of commercial bleach (JIK®). All this was carried out in a clean dust free washing room. The glassware was then dried in the oven at 60oC in a clean dust free place.

Surface sterilization of explants

Young leaves explants were harvested and transported from the greenhouse in a beaker containing tap water to the laboratory. Once in the laboratory, they were cleaned with liquid soap and cotton wool and kept under running tap water for 30Minutes. They were then dipped in 0.5% fungicide (Ridomil)+1DROP0F(2ml)+ savlon (2ml) +2 drpo of Teewn-20 rand kept for 1 hour. The explants were then transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 seconds and rinsed twice with sterile distilled water. The sterilization was carried out using JIK® which contains 3.8% NaOCl (5% and 10%) for different time intervals 5, 10 and 15 minutes. They were then rinsed four times in sterile distilled water. For regeneration of callus, cultures were incubated in a dark room maintained at 25^{0}C.

RESULT AND DISCUSSIONS

Effect of concentration of commercial and exposure time on sterilization.

The present study was conducted to develop efficient sterilization procedure of Anchote young leaf explants. Two different concentration (5% and 10 %) commercial bleach (3.85% NaOCl) of were used for this study with duration of 5, 10 and 15 Minutes leaf plants.

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival (CPRI, 1992). The mean percentage of clean surviving explants showed that the effectiveness of the sterilization procedure increases with 5%commercial bleach and an exposure time of 10 minutes gave significantly higher percentage surviving clean explants (82.5±0.6) than the other concentration and exposure times. Further increase in exposure time led to a significant decline in percentage clean surviving explants.

Table 1: Effect of various concentrations of Commercial Bleach (3.85%NaOCl) and time exposure on leaf explants sterilization of Anchote

Treatments		M%ODE ±SD	M%OFC ±SD	M%OBCE ±SD	M%OCSE ± SD
NaOCl	Time				
0	0	3 ± 0.6^{f}	42 ± 0.5^{a}	30 ± 0.4^{a}	25 ± 0.9^{e}
5	5	4 ± 0.2^{e}	14 ± 0.1^{b}	10.6 ± 0.7^{b}	71 ± 0.3^{bcd}
5	10	6.3 ± 0.2^{d}	5.0 ± 0.3^{c}	6.2 ± 0.3^{c}	82.5 ± 0.6^{a}
5	15	$21.5\pm1b^{c}$	2.7 ± 0.5^{d}	1.5 ± 1^{c}	74.3 ± 0.6^{bc}
10	5	23.1 ± 0.5^{b}	3.6 ± 0.6^{c}	5.2±0.2c	68.0 ± 0.7^{cd}
10	10	25.5 ± 0.6^{b}	2.5 ± 0.5^{c}	2.6 ± 0.2^{c}	69.6 ± 0.7^{cd}
10	15	30.8 ± 0.9^{a}	1.1 ± 0.3^{c}	1.0 ± 0^{c}	67.4 ± 1^{d}

The means followed by the same letters along the columns are not significantly different from each other(α=0.05) Student-Newman-Keuls Multiple Comparisons Tests , SD= Standard **Key**: M%ODE=Mean percentage of dead explants, M%OFCE= mean percentage of fungal contamination explants, M%OBCE= Mean percentage of bacterial contamination explants. M%OCSE = Mean percentage of Cleaning surviving explants.

Table 2 : Effect of various concentrations of BAP and 2,4-D on callus induction from leaf explants of Anchote (Cocinia abyssinica)

Hormones in µM	Percentage of callus responded
BAP +2,4-D	(Mean ±SD)
0+0	0±0e
0+5	65 ± 3^{c}
5+5	80 ± 2^{a}
5+10	77 ± 2^{ab}
5+20	73 ± 2^{b}
0+10	54.3 ± 3^{d}
20+40	67 ± 3^{c}

One-way Analysis of Variance (ANOVA .The means followed by the same letters among the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests , PV≤0.05 ,SD= Standard Deviation.

Table 3: Effect of various concentrations of NAA and 2,4-D on callus induction from leaf explants of Anchote (Cocinia abyssinica).

Hormones in µM	Percentage of callus responded
NAA+2,4-D	(Mean ±SD)
0+0	0+0
0+5	65 ± 3^{c}
5+5	76 ± 3^{a}
5+10	74.4 ± 2^{ab}
5+20	70.5±2b
10+0	43.9 ± 2^{d}
0+40	46.4 ± 1^{d}
20+40	61.3 ± 2^{c}

One-way Analysis of Variance (ANOVA .The means followed by the same letters among the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests , pV≤0.05 ,SD= Standard Deviation.

Plate 3.1.Effects of different concentratio.of auxins and cytokinines on leaf l explants of Anchote callusing: (a) callus induction from explants with 5µM (BAP+2,4-D) ; (b) callus induction from leaf explants with 5µM(NAA+ 2,4-D) and (c). callus induction by 2,4-D only.

At shorter exposure time explants death were due to microbial contamination while at prolonged exposure time death was due to scorching by the sterilants. However, as indicated in the table, at 10 percent of commercial bleach, there was no significant difference in mean percentage of clean surviving explants among the various exposure times. Death was noted to be mainly due scorching by the commercial bleach.

Our present result showed that the leaf explants of Coccinia abyyssinica have shown different sterility response for different concentration of bleach at different time duration. This shows that different tissue type requires different concentration of sterilants and time exposures. This result agreed with (Rezadost et al, 2013) who reported that the surface sterility chosen for an experiment typically depend on the explants and also plant species. The present study also demonstrates that commercial bleach effective to remove surface contaminants from Anchote leaf explants. Various research work shown that different explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field-grown tissues. The time of sterilization is dependent on the type of tissue; for example, leaf tissue require a shorter sterilization time than seeds with a tough seed coat.

(Funguomali et al, 2013, Sharma and Nautiya, 2009). Our result for the present sterilization study also conform that leaf l explants responded for different concentration of commercial Jik® and duration time.

Effect of concentration Hormones on callus induction

Plant hormones (phytohormones) are small organic molecules that affect diverse developmental processes. Alterations in hormone responses have been responsible for several important agricultural advances, such as the breeding of semi-dwarf varieties and increased grain production (Ashikari et al. 2005). Unlike animal hormones, which are produced in specific organs, phytohormones are typically produced throughout the plant Virtually every aspect of plant development from embryogenesis to senescence is under hormonal control. In general, this developmental control is exerted by controlling cell division, expansion, differentiation and cell death. In this manner, diverse developmental processes can be controlled, including formation of the apical-basal and radial pattern, seed germination, shedding(Silverstone and Sun, 2000).

Callus is an unorganized mass of cells that develops when cells are wounded and is very useful for many in vitro cultures. Callus is developed when the explant is cultured on media conducive to undifferentiated cell production—usually the absence of organogenesis (organ production) can lead to callus proliferation (Mungole et al.,2011) . Once callus begins to form organs, callus production is halted. Auxins and cytokinins both aid in the formation of most callus cells(Ali et al., 2007).

In our present investigation callus in induction were directly initiated from young leaves explants of Anchote (Coccinia abyssinica) after successful sterilization. The young leaves explants were transferred to fresh MS medium supplemented with different concentration of auxins and cytokinins including (2,4-D, BAP, NAA) of which the most effective one's are given in (Table 3.2 and table3.3). On MS basal medium supplemented with 2,4-D (5µM) of the leaf explants showed callus initiation after 11th day of inoculation from basal cut portion of leaf with mean percentage (65±3) cultures. On MS basal fortified with NAA and 2,4-D(5+5)µM, the leaf explants exhibited callus initiation with maximum mean percentage of (76±3) cultures up to 10 days of inoculation. The callus responding increased in size in the same media composition up to 4 weeks of inoculation for leaf explants.

MS basal medium supplemented with BAP+2,4-D (0+5, 5+5, 5+10, 0+10, 20+40) µM induced callus in different cultures after 10th day of inoculation as shown in (Table3.2). On other hand , On MS basal medium supplemented with BAP+2,4-D, the percentage of callusing was increased with an increasing in concentration up to 5 µM and then declined with a further increase in the concentration of BAP+2,4-D(20+ 40)µM . This was gave minimum mean percentage (54±2) and this significantly different at $p<0.05$.The optimum concentration of BAP + 2, 4-D in terms of maximum mean percentage of callusing (80±2) was observed .

from leaf explants at 5.0 µM when BAP+2,4-D were used in combination (Plate3.1).But the lower mean percentage of calli were obtained from highest concentration of the hormones for explants (Table3.2). Therefore, the combination of 2,4-D and BAP gave high frequency of callusing over combination of NAA and 2,4-D. Our result is supported by

(Devendra et al, 2009) who found that combination of BAP with 2,4-D or NAA produced callus in cucumber.

CONCLUSION

In general, Sterilization should therefore guarantee the explants sterility and regeneration capacity which are known to be affected by disinfectant concentration and sterilization period. The in vitro protocol reported in this study can be used for sterilization of explants from vegetative tissues, rapid multiplication of disease free plants, sustainability of genetic modification and transformations leaf explants of Anchote (Coccinia abyssinca) and other related species .From the above study, it is also concluded that the callus induction in Anchote (Cocinia abyssinica) were established from leaf region of the plant on MS medium supplemented with 2,4-D, NAAand BAP.

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