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In Vitro Direct Organogenesis Protocol for Mass Propagation of an Elite Ethiopian Hot Pepper (*Capsicum annuum* L.) Cultivar: Mareko Fana

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Abstract

An efficient and reproducible *in vitro* mass propagation protocol was developed for Mareko Fana cultivar of hot pepper (*C. annuum* L.) through direct organogenesis using nodal and shoot tip explants. Three percent active chlorine for 20 minutes was found to be optimum treatment combination yielding 82.5% ± 5.00% contaminant-free germinated seedlings. For shoot induction, MS + 4.5 mg/l BAP + 0.5 mg/l IAA and MS medium containing eight mg/l Zeatin were found to be optimum resulting 77.5% ± 5.00% and 67.50% ± 5.00% induction percentage for nodal and shoot tip explants respectively. Maximum shoot multiplication responses were obtained on MS + 3 mg/l BAP + 2 mg/l Kinetin with mean number of 9.2 ± 0.2 and 8.6 ± 0.00 shoots for nodal and shoot tip explants respectively. Best shoot elongation and rooting responses were obtained on MS + 0.5 mg/l IBA resulting mean value of 29.6 ± 0.12 root number, 4.25 ± 0.20 cm root length and 5.12 ± 0.20 cm shoot height. The plantlets showed 77.5% survival during acclimatization and transplanting.

Keywords

Capsaicin, Micro Propagation, Micro Cuttings, Micro Shoots, Pepper, Plant Growth Regulators

1. Introduction

Hot pepper (*Capsicum annuum* L.) is an herbaceous plant that belongs to the genus *Capsicum* of the night shade family, Solanaceae. Commercially, *Capsicum* peppers are classified by the concentration of *capsaicin* ($C_{18}H_{27}NO_3$)

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which determines a variety's "hotness" [1]. Fruits of *Capsicum* plant rank among the most heavily valued and consumed species throughout the world due to their unique flavor and pungency. The fruits are consumed fresh, as vegetables, spices or condiments and dried or processed products to meet diverse end uses [2] [3]. The world demand for *Capsicum* has been continuously increasing recently, and production increased by 40% between 1990 and 2000 with about 1.4 million hectares cultivated [4]. Asian production of *Capsicums* accounts for 56.4% of world production, followed by the southern European region with 15% of world production in 2000. Africa does not produce as many fresh *Capsicums* as other regions, and uses most of the total production for dried pepper powder. Currently China is the main producer and exporter in the world. The top importers are USA, Germany, Spain and Japan [5].

In Ethiopia *Capsicums* have been grown for a long time by local farmers and considered as an indigenous vegetable crop because for most Ethiopians food is tasteless without hot pepper. Pepper generated an income of 509.44 million birr for small holder farmers in 2004/05 [6] [7]. Further, the crop is the main industrial raw material for processing of capsaicin and color oleoresins [8]. Pepper grows under warm and humid weather conditions [9], and it is extensively grown in most parts of the country, with the major production areas concentrated at altitude of 1100 to 1800 meter above sea level [10]. A great deal of natural hybridization has occurred among species and many local genotypes have evolved with various plant and fruit characters [11]. Research on *capsicum* started with minor observation and mass selection from local materials in different experimental stations of Awasa and Bako Research Centers [12]. Mareko Fana is a local cultivar developed by mass selection for its larger and pungent pods with highly demanded dark-red color, and is recommended for cultivation in most of the Ethiopian regions. Mareko Fana is the only cultivar being used for a long time by the local factories for the extraction of capsicum oleoresin for the export market [8] [13].

Hot pepper plant is conventionally propagated by seed and quality of seed is a crucial factor for good pepper production [14]. The conventional propagation method, however, has disadvantages, including genetic variability, short viability period, low rate of germination, and high risk of infection by various diseases and pests. In addition, since the plant also lacks natural vegetative propagation, tissue culture methods provide a novel way for the asexual multiplication of hot pepper plants [15]. Micro-propagation enables a rapid rate of clonal multiplication of an elite plant species, allowing production of disease-free, genetically stable and uniform progenies [16] [17]. *In vitro* procedures for multiplication of *C. annuum* cultivars using different explants have been reported by various authors [15] [18]-[20]. However, most of the reports on response of hot peppers suggest a strong influence of genotype on the regeneration process [21]. So, this research work was initiated to develop an efficient and reproducible protocol that enables rapid *in vitro* multiplication of Mareko Fana cultivar of *C. annum* from shoot tip and nodal explants.

2. Materials and Methods

Healthy and normal seeds of fresh ripe fruits obtained from Bako Agricultural Research Center (BARC) were used as a starting material and stored under optimum conditions until the experiments were started.

Media used in the experiments were MS [22] basal media supplemented with 30 mg/l sucrose, the Cytokinins; BAP, Zeatin and Kinetin, and the Auxins; IAA and IBA. The pH of the media was adjusted to 5.8. In all cases, cultures were maintained in a relatively controlled growth rooms at 27˚C ± 2˚C temperature and light regimens of 1000 - 2000 lux light intensity from cool white florescent lumps of 40 W at a 12 - 12 h light-dark cycle.

For initiation of aseptic culture seeds were taken out of plastic bags using forceps and washed three times with tap water and commercial liquid detergent. Then after, the seeds were kept under running water for 10 minutes. The cleaned seeds were then transferred to the laminar flow hood, immersed in 70% (v/v) ethanol for 1 minute and rinsed three times in sterile distilled water. This was followed by surface sterilization with 2% - 4% active chlorine in NaOCl solution for 10 - 25 minutes. The solution was decanted and the seeds were rinsed five times with sterile distilled water. They were then cultured in test tubes of PGR-free MS basal media containing 3% sucrose for germination. From 35 days old seedlings grown *in vitro*, 1 - 2 cm. long nodal and 1 - 1.5 cm long shoot tip explants were trimmed and transferred on to shoot induction media.

This experiment was a 3 × 4 factorial combination in CRD. The two factors were three concentrations (2%, 3%, and 4%) of active chlorine and four durations (10, 15, 20 and 25 minutes) of explants exposure.

For shoot induction and multiplication, shoot apices (1 - 1.5 cm) and nodes (1 - 2 cm) were taken from five weeks old *in vitro* germinated seedlings and cultured on agar solidified (0.7% agar-agar) MS basal medium sup-

plemented with different concentrations (1.5, 3, 4.5, 6 mg/l) of BAP and (4, 8, 12 mg/l) Zeatin alone, and in combination with 0.5 mg/l and 1 mg/l IAA each. Therefore, the experiment was laid out with a treatment combination of four concentrations of BAP, three concentrations of Zeatin and two concentrations of IAA for shoot tip and nodal explants in CRD design in 4 × 3 × 2 × 2 factorial combination.

Micro shoots, responded well, were transferred singly onto a shoot multiplication medium. The media contained BAP (1.5, 3.0, 4.5, 6.0 and 7.5 mg/l), Kinetin (1.0, 2.0 and 3.0 mg/l) each alone and interacting one another at each level, and each levels of BAP combined with 0.5 mg/l IAA. The experiment was arranged in a 5 × 3 × 2 factorial in CRD. After three weeks, cultures proliferating shoot clumps were divided and five each per jar were sub cultured on to a fresh medium of similar composition.

Elongated micro cuttings (about 2 cm long) obtained from shoot tip and nodal cultures were excised and cultured on media supplemented with (0.25, 0.5, 0.75 and 1 mg/l) IAA and IBA. Therefore, the experiment was laid out in CRD design in 4 × 4 factorial combinations.

The elongated shoots with well developed roots were taken for acclimatization *ex vitro* and transferred to a sterilized potting mix of forest soil: sand: well decomposed coffee husk at a respective (v/v) ratio of 1:1:2 and kept under a plastic tunnel of high humidity (80% - 90%) to prevent desiccation, for ten days. In the tunnel, water was sprayed everyday to maintain the RH (relative humidity) as high as possible, to simulate the RH of the culture bottles. Starting from the seventh day, the RH within the tunnel was reduced gradually to reach about 60% at the end of the tenth day. After the tenth day, the plantlets were transferred to a 70% shade net, where they were retained for another one month. Later, they were transferred to a 30% shade net and maintained for another month, prior to field transplanting.

3. Experimental Design, Data recording and Data Analysis

All experiments were laid in CRD with factorial treatment combinations, having 20 replications per treatment. All the experiments were repeated two times to ensure reproducibility of the results and the average were considered for analysis.

After sterilization experiment, the number and percentage of contaminant free explants and tissue death was recorded during the first two weeks of seed culture. For the second experiment, the percentage of explants forming shoot buds were counted after four weeks of culture while the number of shoot buds at each sub culture were recorded for experiment three and the mean value taken for analysis. The number of roots, root length and the length of the shoots was recorded after five weeks of culture.

Average of the data from the two repetitions for each experiment were subjected to statistical analyses using the SAS statistical software (Version 9.2) and ANOVA was constructed, followed by mean separation using appropriate procedures (REGWQ). When the ANOVA indicated significant treatment effects (5%, 1%, or 0.1%) based on the F-test, probability level of 0.05 ($P \leq 0.05$) was used to determine which treatments were statistically different from the other treatments.

4. Results and Discussion

The ANOVA obtained from the data showed that the concentration of active chlorine, time duration of explants exposure to the sterilant and interaction of sterilant concentration to time duration had very highly significant effect ($P < 0.0001$) on the removal of contaminant agents from *C. annum* seed explants.

The least percentage of clean culture (7.5%) and the highest level of contamination (90%) were seen from explants treated with two percent active chlorine for ten minutes (**Table 1**). Increasing the sterilant concentration from two to four percent maintaining the 10 minute exposure duration constant had reduced the rate of contamination to 22.5%. However, only 55% of the seed explants survived to germinate on the culture media. This effect, though not exclusive, was attributed to tissue death (22.5% ± 5.00%) caused by the very high concentration of sterilant chemical. Time exposure duration on the other hand had significantly affected the effectiveness of the sterilant concentration that the increase in time from 10 minute to 25 minute at four percent active chlorine had decreased the percentage of clean culture from 55% to 20% (**Table 1**) increasing the level of tissue death from 22.5% to 77.5%.

The highest percentage of contaminant free seeds and hence germination (82.5% ± 5.00%) was achieved at a relatively medium level of sterilant concentration (3% active chlorine) and optimum time of exposure duration (20 minute) with only about 10% contamination level and 7.5% tissue death (**Table 1**).

Table 1. Interaction effect of different concentrations of sodium hypochlorite and time of exposure on removal of contaminant factors from hot pepper seed explants.

% of Active Chlorine in NaOCl Solution	Exposure Duration of Explants (minute)	N	% Clean Culture
			Mean ± Std Dev
0	0	4	0.00 ± 0.00
2	10	4	7.50^{h} ± 5.00
2	15	4	20.00^{g} ± 0.00
2	20	4	42.50^{de} ± 5.00
2	25	4	47.50^{cde} ± 5.00
3	10	4	40.00^{e} ± 0.00
3	15	4	55.00^{c} ± 5.77
3	20	4	82.50^{a} ± 5.00
3	25	4	70.00^{b} ± 0.00
4	10	4	55.00^{c} ± 5.77
4	15	4	50.00^{cd} ± 0.00
4	20	4	30.00^{f} ± 0.00
4	25	4	20.00^{g} ± 0.00

R-Square = 0.982143; Coeff Var = 8.951436; Means with the same letter in a column are not significantly different at Alpha = 0.05.

Generally, occurrence of high contamination rate of explants in a relatively lower sterilant concentration and shorter exposure time treatment combination was possibly due to the insufficiency of sterilant concentration and exposure duration to remove or kill the contaminant microbes or the fungal and bacterial contaminants survived the given treatment combination. The higher the concentration of active chlorine and longer exposure duration resulted better removal or death of microbes due to the powerful oxidant property of active chlorine that disintegrates the lipids in the cell walls of bacteria and fungi. The effect of sterilant chemical could also alter or denature the shape and function of microbial enzymes [23]. However, the increase in sterilant concentration and exposure time above certain optimum limit causes loss of explants because of the oxidant chemical ingredient of the sterilant solution killing the embryo of the seed as well. Hence, the optimum concentration and exposure duration for effective sterilization of explants should be determined based on two aspects of the observations, i.e. a relatively minimum level of contamination with a relatively minimum level of explants tissue death which gives the maximum percentage of clean lively culture.

This result is partially in agreement with [19] who reported a minimum level (15%) of pepper seed explants contamination using four percent active chlorine in sodium hypochlorite solution for 20 minute. The use of 0.1% mercury chloride for five minute was reported as effective treatment combination for sterilization of *C. annum* seed explants on initiation of aseptic culture *in vitro* by some authors. The use of mercury chloride now a day's however, is highly discouraged as such chemicals have been known to have a seriously harmful residual effects to both human and the environment [24]. Thus, the trend is to try for the replacement of safe and relatively environmentally friendly or harmless chemicals such as sodium hypochlorite solution as employed in the present study.

Aseptic shoot tips and nodal explants excised from lateral branches of *in vitro* grown seedlings (**Figure 1(a)**) were transferred on MS media containing different concentrations of Zeatin (4 - 12 mg/l), BAP (1.5 - 6 mg/l) or the combination of BAP (1.5 - 6 mg/l) and IAA (0.5 and 1 mg/l) for four weeks to determine optimum medium for shoot induction of *C. annum* L., Mareko Fana cultivar.

Very highly significant effect ($P < 0.0001$) of BAP and Zeatin, explants type and interaction of BAP with IAA to the rate of shoot induction was revealed from the ANOVA output. Interaction effect of explants type by BAP with IAA on rate of shoot induction was found to be highly significant (BAP*IAA*Expl, $P = 0.0003$). This ANOVA product is an indication of the interaction among BAP and IAA to be explants dependent on *in*

(a) (b) (c) (d) (e)

Figure 1. (a) An *in vitro* germinated seed of *C. annum* L. on PGR free MS medium after two weeks; (b) Micro shoots multiplied on 3 mg/l BAP + 2 mg/l kinetin after three weeks; (c) Elongated and rooted plantlet on MS + 0.5 mg/l IBA after three weeks; (d) Bottom view of long and thin roots with fine root hairs developed on MS + 0.5 mg/l IBA; (e) Hardened and acclimatized plantlets transferred to pot.

vitro shoot induction of Mareko Fana cultivar of *C. annum* L. The response of shoot tip and nodal explants to a given level of BAP and IAA combination was not the same that, the nodal explants gave greater response than shoot tip explants. This might be related to the variation in endogenous level of auxin in shoot tip and nodal sections of the given genotype [25].

Shoot bud regeneration capacity of shoot tip and nodal explants increased with increase in concentration of both BAP and Zeatin from zero to 4.5 mg/l and from zero to eight mg/l respectively, and reduced with further addition of both BAP and Zeatin (**Table 2**). This could be due to the exogenous application of cytokinins which release the shoot buds from apical dominance. Addition above certain optimum level however, reduced shoot induction rate by inhibiting the availability of required endogenous amount of auxin for shoot bud proliferation as cytokinins do not act alone unless together with auxins.

Although it has been repeatedly reported that exogenous auxin does not promote axillary shoot proliferation, a low concentration of auxin together with a relatively high level of cytokinin is often useful during shoot multiplication [23] [25]. Such an effect was observed in the present study that, the combination of BAP and IAA produced best response. When a relatively low concentration of IAA is combined with high BAP than both increased or both decreased (**Table 2**). The maximum rate of shoot induction was achieved on an MS medium supplemented with the combination of 4.5 mg/l BAP and 0.5 mg/l IAA (77.5% ± 5.00%) and Ms medium containing eight mg/l Zeatin (67.50% ± 5.00%) for nodal and shoot tip explants respectively (**Table 2**). The second best ranked media for effective shoot bud induction was found to be MS medium added with eight mg/l Zeatin (70% ± 0.00%) and 4.5 mg/l BAP alone (62.50% ± 5.00%) for nodal and shoot tip explants respectively.

The combination of 1.5 mg/l BAP and 1 mg/l IAA produced no response of shoot bud induction rather than callus formation while the same level of BAP with 0.5 mg/l IAA gave very low response (10% - 20%) for both shoot tip and modal explants. Increasing the level of BAP to 4.5 mg/l keeping the 0.5 mg/l IAA constant however, had shown a great deal of improvement in shoot induction as discussed above. This backs the knowledge that, it is the ratio of auxin to cytokinin, not the absolute level of auxin that suppresses shoot bud growth [25].

This result is the same with [15] that reported maximum shoot bud induction response (75%) of *C. annum* nodal explants on MS medium fortified with 5 mg/l BAP in combination with 0.5 mg/l IAA. It also partially agrees with [20] who reported a maximum rate of shoot bud induction (80%) from shoot tip explants of *C. annum* genotypes on MS medium containing 10 mg/l Zeatin. The effectiveness and best response of pepper nodal explants to *in vitro* shoot induction on MS medium containing 2 mg/l BAP combined with 0.5 mg/l IBA has been reported by [19]. In the present study, however, both 1.5 mg/l BAP combined with 0.5 mg/l IAA and 3 mg/l BAP with 0.5 mg/l IAA for shoot tip and nodal explants studied, the rate of shoot bud induction was very low.

The ANOVA revealed that the level of BAP both alone and together with Kinetin and IAA had very highly significant effect ($P > 0.0001$) on shoot multiplication rate. The application of Kinetin alone had no significant effect unless combined with BAP. Shoot buds raised from both shoot tip and nodal explants responded almost similarly on shoot multiplication indicating the non-significant effect ($P = 0.0691$) of explants at 5% probability level. The proliferation rate had shown progressive increase from the first sub cultures to the second sub culture by an average of 33.3%. In this study however, further steps of sub cultures that enables the examination of the overall progress up to declining stage was not carried out.

Various genotypes of *C. annum* L. required different optimum concentration of BAP, Zeatin and Kinetin

Table 2. Effect of Zeatin, BAP and combination of BAP with IAA on shoot induction of *C. annum*.

Media	Plant Growth Regulators			Shoot Bud Induction (%)	
	ZEA (mg/l)	BAP (mg/l)	IAA (mg/l)	Node (Mean ± S.D)	Shoot Tip (Mean ± S.D)
MS1	4	-	-	$40.00^{cd} \pm 0.00$	$27.50^{f} \pm 5.00$
MS2	8	-	-	$70.00^{a} \pm 0.00$	$67.50^{a} \pm 5.00$
MS3	12	-	-	$50.00^{b} \pm 0.00$	$40.00^{d} \pm 0.00$
MS4	-	1.5	-	27.50 ± 5.00	$22.50^{f} \pm 5.00$
MS5	-	3	-	$45.00^{cd} \pm 5.77$	$40.00^{d} \pm 0.00$
MS6	-	4.5	-	$67.50^{a} \pm 5.00$	$62.50^{ab} \pm 5.00$
MS7	-	6	-	40.00 ± 0.00	$37.50^{e} \pm 5.00$
MS8	-	1.5	0.5	$17.50^{g} \pm 5.00$	$12.50^{g} \pm 5.00$
MS9	-	3	0.5	$45.00^{cd} \pm 5.77$	$37.50^{e} \pm 5.00$
MS10	-	4.5	0.5	$77.50^{a} \pm 5.00$	$60.00^{ab} \pm 0.00$
MS11	-	6	0.5	$52.50^{b} \pm 5.00$	$40.00^{d} \pm 0.00$
MS12	-	1.5	1	0.00 ± 0.00	0.00 ± 0.00
MS13	-	3	1	$35.00^{e} \pm 5.77$	$32.5^{e} \pm 5.00$
MS14	-	4.5	1	$57.50^{ab} \pm 5.00$	$45.00^{cd} \pm 5.77$
MS15	-	6	1	$45.00^{cd} \pm 5.77$	$40.00^{d} \pm 0.00$
Control	-	-	-	0.00 ± 0.00	0.00 ± 0.00

Means with the same letter in a column are not significantly different at Alpha = 0.05.

alone and in combination with IAA or IBA [15] [18]-[20]. In this study the best result of shoot proliferation (9.20 ± 0.2 and 8.6 ± 0.00) was obtained on MS medium containing 3 mg/l BAP combined with 2 mg/l Kinetin (**Figure 1(b)**) for nodal and shoot tip explants (**Table 3**) with no significant difference to the explants type from which the shoot buds were raised. This has not been reported by most of the published works on *in vitro* propagation of *C. annum* genotypes. The second ranking shoot number (7.26 ± 0.11) and (7.06 ± 0.11) was obtained on MS medium added with 6 mg/l BAP together with 0.5 mg/l IAA for shoot buds raised from nodal explants and shoot tip explants respectively (**Table 3**). This result is in line with [15] and [19] who reported shoot number of 8.8 ± 0.15 and 7 ± 0.17; 6.3 ± 0.1 and 4.7 ± 0.39 for three different genotypes on MS medium containing 5 mg/l BAP together with 0.5 mg/l IAA or IBA respectively. Cultures on high (7.0 mg/l and above) cytokinin media had proliferated bushy and ill defined shoot buds and they did not respond when sub cultured on the same medium. The absence of shoot multiplication response on medium containing kinetin alone has also been reported by [20] [26] [27].

An increase in number of shoots per shoot bud culture with increased concentration of BAP from zero to 6 mg/l (**Table 3**) might be due to the effect of BAP (cytokinin) in releasing lateral buds from dormancy or breaking apical dominance by inhibiting the level of endophytic auxins. The later effect might also be attributed to the reduction in number of shoots with further increase of BAP to 7.5 mg/l by removing the required minimum amount of auxin [23] [25].

Micro shoots of 1.5 - 2.5 cm height were taken from the prevailing medium without discrimination between the explants type from which they were raised and cultured on MS media fortified with (0.25 - 1 mg/l) IAA and IBA for five weeks in order to determine the most effective medium composition for *in vitro* root induction of *C. annum* L., Mareko Fana cultivar.

In this result, both IBA and IAA were found to be effective in promoting root induction at optimum level. About 95% of shoots were rooted after two weeks of culture at 0.25 and 0.5 mg/l concentration of both IBA and IAA. The highest number and length of root and shoot however, was achieved on MS media containing IBA than IAA (**Table 4**). The roots induced on media containing IBA were normal, thin and long with many

Table 3. Effect of interactions among PGRs on *in vitro* shoot multiplication of *C. annum.*

Media Type	Plant Growth Regulators (mg/l)			Number of Shoot per Explant (Mean ± S.D.)	
	BAP (mg/l)	KINETIN (mg/l)	IAA (mg/l)	Node	Shoot Tip
Control	-	-	-	0.00 ± 0.00	0.00 ± 0.00
MS1	-	1	-	0.00 ± 0.00	0.00 ± 0.00
MS2	-	2	-	0.00 ± 0.00	0.00 ± 0.00
MS3	-	3	-	0.00 ± 0.00	0.00 ± 0.00
MS4	1.5	-	-	1.66^{p} ± 0.11	1.00^{p} ± 0,00
MS5	3	-	-	2.53^{lm} ± 0.11	1.00^{p} ± 0,00
MS6	4.5	-	-	4.86^{f} ± 0.23	4.66^{f} ± 0.11
MS7	6	-	-	3.73^{i} ± 0.11	3.00^{k} ± 0,00
MS8	7.5	-	-	3.2^{k} ± 0.2	2.8^{kl} ± 0.00
MS9	1.5	1	-	2.06^{o} ± 0.11	2.00^{o} ± 0,00
MS10	1.5	2	-	2.8^{l} ± 0.20	2.66^{l} ± 0.11
MS11	1.5	3	-	3.73^{i} ± 0.23	3.66^{j} ± 0.11
MS12	3	1	-	6.26^{cd} ± 0.11	6.06^{d} ± 0.11
MS13	3	2	-	9.20^{a} ± 0.20	8.6^{a} ± 0.00
MS14	3	3	-	6.6^{c} ± 0.00	6.2^{c} ± 0.20
MS15	4.5	1	-	5.33^{e} ± 0.11	5.2^{e} ± 0.00
MS16	4.5	2	-	4.93^{f} ± 0.11	4.73^{f} ± 0.11
MS17	4.5	3	-	4.60^{f} ± 0,00	4.26^{g} ± 0.11
MS18	6	1	-	4.33^{g} ± 0.23	4.2^{g} ± 0.20
MS19	6	2	-	4.00^{h} ± 0,00	4.06^{h} ± 0.11
MS20	6	3	-	3.73^{i} ± 0.11	3.93^{i} ± 0.11
MS21	7.5	1	-	3.46^{j} ± 0.11	3.40^{j} ± 0.00
MS22	7.5	2	-	3.00^{k} ± 0.00	3.20^{k} ± 0.00
MS23	7.5	3	-	2.20^{n} ± 0.00	2.33^{n} ± 0.11
MS24	1.5	-	0.5	0.00 ± 0.00	0.00 ± 0.00
MS25	3	-	0.5	3.13^{k} ± 0.11	2.66^{l} ± 0.111
MS26	4.5	-	0.5	6.40^{c} ± 0.00	$6.13^{d.}$ ± 0.11
MS27	6	-	0.5	7.26^{b} ± 0.11	7.06^{b} ± 0.11
MS28	7.5	-	0.5	4.86^{f} ± 0.11	5.06^{ef} ± 0.11

Means with the same letter in a column are not significantly different at Alpha = 0.05.

Table 4. Effect of different concentrations of IAA and IBA on *in vitro* root induction of *C. annum*.

Media Type	Plant Growth Regulators (mg/l)		Mean Number of Root per Microcutting	Mean Root Length (cm)	Mean Shoot Length (cm)
	IBA	IAA			
MS1	0.25	-	18.3^{b} ± 0.31	3.30^{b} ± 0.11	3.41^{c} ± 0.31
MS2	0.5	-	29.6^{a} ± 0.12	4.25^{a} ± 0.20	5.12^{a} ± 0.20
MS3	0.75	-	13.4^{c} ± 0.02	3.23^{b} ± 0.31	3.36^{c} ± 0.12
MS4	1.0	-	7.66^{e} ± 0.04	3.19^{b} ± 0.13	3.28^{c} ± 0.14
MS5	-	0.25	10.04^{d} ± 0.03	3.00^{c} ± 0.00	2.79^{d} ± 0.05
MS6	-	0.5	17.50^{b} ± 0.01	3.71^{a} ± 0.07	4.10^{b} ± 0.04
MS7	-	0.75	8.33^{e} ± 0.21	2.72^{d} ± 0.02	2.97^{d} ± 0.17
MS8	-	1	5.16^{f} ± 0.02	2.44^{e} ± 0.20	2.83^{d} ± 0.23
Control	-	-	0.00 ± 0.00	0.00 ± 0.00	2.44^{e} ± 020

Means with the same letter in a column are not significantly different at Alpha = 0.05.

branches and root hairs (**Figure 1(d)**). The maximum number of roots (29.6 ± 0.12), the highest root length (4.25 ± 0.20) and the highest shoot height (5.12 ± 0.20 cm) was recorded on 0.5 mg/l IBA (**Figure 1(c)**; **Table 4**). Mean number of 17.50 ± 0.01 roots, 3.71 ± 0.07 root length and 4.10 ± 0.04 cm shoot height was attained on the same concentration of IAA as the best result (**Table 4**). This result is in accordance with or the same to those reported by [15] and [19] and [20] which evidenced 0.5 mg/l IBA as the best hormonal type and level for effective *in vitro* rooting of different *C. annum* genotypes.

The reduction in rooting response with the increase in auxin (both IAA and IBA) from 0.5 to 1 mg/l might be due to the high concentration of auxin in the range that normally stimulates elongation of shoots (10^{-5} to 10^{-6} M), cause a significant inhibition of root growth. The inhibition of root growth and development might partly be due to ethylene production which is triggered by high auxin concentration [24] [25].

In the present study, the regenerated plants showed 77.5% survival efficiency. There were no observable variations with respect to morphological and growth characteristics between *ex vitro* sown parent plants and *in vitro* raised plants in pots. The transplanted plants had established well in pots (**Figure 1(e)**).

5. Conclusion

Thus, this protocol could be useful for large scale production of Mareko Fana cultivar, *C. annum* L., provide a possible system towards genetic improvement of the crop and increase the efficiency of transformation protocols using nodal as well as shoot tip explants sources.

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