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In vitro: Influence of Various Concentrations of Plant Growth Regulators (BAP & NAA) and Sucrose on Regeneration of *Chenopodium quinoa* Willd*.* Plant

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Authors' contributions

This work was carried out in collaboration between both authors. Authors FRAG and HEAES designed the study, wrote the protocol, initiated the experiments, collected the data, performed the statistical analysis, managed the literature review and wrote the final draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

In vitro: regeneration of *Chenopodium quinoa Willd.* was achieved from cotyledonary nodes explants. In this study, used 6-Benzylaminopurine (BAP) and α-Naphthalene Acetic Acid (NAA) of plant growth regulators with different concentrations individually as well as in combination and used different concentrations of sugar (sucrose) with different concentrations. For was rooting, used half strength (½MS), full-strength MS and ½ MS supplemented with 0.2 mg/l of NAA. The results mentioned, explant responding (%) to multiplication was about 73% for all BAP treatments compared with control and average numbers of shoot increased with increased BAP concentration except 5 mg/l of BAP. The highest explant responding (%) was in media supplemented BAP without NAA compared other treatments noted that the media with combination of BAP and NAA gives formation of callus in bases of the plantlets. Also, the result inducted the combinations between

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(BAP–NAA) was highly significantly (*P≤ 0.001*) and less effective on number of shoots where the highest number of shoot was 3.40 in media with 3 mg/l BAP compared other treatments. The highest of explant responding 93.33% was in media supplement with 10 g/l sucrose and (10 g/l sucrose + 3 mg/l BAP), but sucrose level for good greening and developed shoots (4 shoots) was in medium supplement with 10 g/l sucrose. The shoots rooted well on half-strength MS medium with 60% percentage of root. The rooted shoots were acclimatized and transferred to green house to follow their development.

Keywords: Regeneration; sucrose; cotyledonary node; plant growth regulators (BAP; NAA); ½ MS; MS.

ABBREVIATIONS

6-Benzylaminopurine (BAP), α-Naphthalene Acetic Acid (NAA), 6-Benzyladenine (BA), Germination Rate, Murashige and Skoog medium (MS), half strength Murashige and Skoog medium (½ MS), Plant Growth Regulator (PGR).

1. INTRODUCTION

1.1 Quinoa Plant

Chenopodium quinoa Willd., belonging to the C_3 group of plants and it is an annual herbaceous, dicotyledonous crop species and referred as a pseudo-cereal plant of the family Chenopodiaceae, but since 2009, phylogenetic classification ranks quinoa in the family Amaranthaceae [1]. High-nutrition content for quinoa makes it an ideal candidate for supporting growing populations such as Africa and Asia where quinoa contains 55.3 % carbohydrates, 12.4 % lipids, and 11.7 % proteins [2]. In 2013, the United Nations Assembly declares "International Year of Quinoa", aware Quinoa is important, where contains all the main amino acids and several important trace elements and vitamins needed for human life [3].

1.1.1 Scientific classification

Quinoa consider halophyte crop and grown in the Andean highlands at an altitude of 3500–3900 m above sea level, that exposed frequently to drought, frost, wind, hail and soil salinity as shown in Fig. 1 [4-7]. However, It adapts to climate temperatures a range from 4°C to 38°C, from dry climates to relative humidity's of 88% [8].

The chemical constituents in quinoa seed and their therapeutic properties, representing this crop an important resource for the development of functional foods. In addition, some bioactive compounds of Quinoa have shown interesting pharmacological properties, suggesting possible applications in the medicinal field. Moreover, Quinoa consider food alternative for people suffering from celiac disease, because it does not contain gluten [9].

Classification of cronquist (1981)	
Kingdom	Plant
Division	Magnoliophyta
Classe	Magnoliopsidae
Sous-classe	Caryophyllidae
Ordre	Caryophyllales
Famille	Chenopodiaceae
Genre	Chenopodium
Classification APG III (2009)	
Ordre	Caryophyllales
Famille	Amaranthaceae

1.2 Plant Growth Regulators (PGRs)

Plant Growth Regulators (PGRs) play an important role in determining the development pathway of plant cells and tissues in culture medium. The most commonly of PGR's are auxins, cytokinins and gibberellins. The type and the concentration of hormones used depend on the species of the plant, the tissue or organ cultured and the objective of the experiment [10]. The most widely used of plant growth regulators in plant tissue culture are auxins and cytokinins and their amount determined the type of culture established or regenerated [11]. Most commonly used cytokinins are 6-Benzylaminopurine (BAP), 6-Benzyladenine (BA), Kinetin, and Zeatin. These hormones, are involved with cell division, modification of apical dominance, shoot differentiation etc. But, they usually promote cell division if added together with an auxin. BAP is the most effective cytokinins for stimulating axillary shoot proliferation [12]. Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) are the most

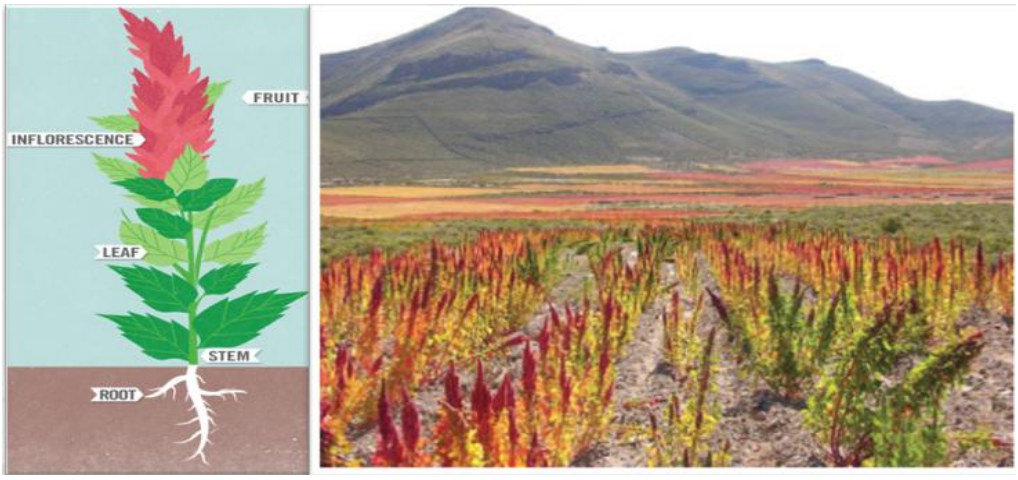

Fig. 1. *Chenopodium quinoa Willd.* plant

used auxin in plant tissue culture. Generally, auxins are involved in cell division, elongation, cell wall synthesis and rooting [12]. Hausman [13] has founded that in tissue culture media, IAA is photo-oxidized rapidly (50 % in 24 h) while IBA oxidized slowly (10 %) and NAA is very stable.

Usually, sugar (sucrose) used as source for carbon in media and it has important role in shoots regeneration, while is this study try to investigate the best media for regeneration of quinoa plant. Currently, there are not regeneration protocols adapted to quinoa by cotyledonary nodes explants. The aim of this work was to develop a complete regeneration method for quinoa, and the influence of PGRs (BAP & NAA) and/or sugar (sucrose) with different concentrations *in vitro*.

2. MATERIALS AND METHODS

2.1 Plant Material and Culture Media Conditions

The quinoa (*Chenopodium quinoa* Willd.) seeds were collected from Living Now Company from USA and sterilized through dipped into 70% ethanol for 15 second, then washing its by sterile distilled water for many times to get rid of alcohol residue. After that, seeds were surface sterilized for 20 min. in 10% sodium hypochlorite NaOCl, then rinsing for it's multiple times with sterilized distilled water, (this was done under a laminar flow hood), the culture media which used in all experiments were MS [14]. Before media autoclaving at 121°C for 20 min, pH adjusted to 5.6 by NaOH (1N) or HCl (1N) with adding 30 g/l sucrose and solidified with 6 g/l agar.

2.1.1 Germination seeds

For germinations, the sterilized seeds were transferred to petri dishes containing 20 ml media (10 seeds per petri dish). The cultures were incubated in growth chamber under light (12 hr/ day) condition where the fluorescent light intensity was 1000 Lux and temperature was maintained at 25±2 °C. Then, Germination rates (%) (GR %) germination rate was calculated by the following formulae after 15 days.

(GR %) = (Number of Germinated Seeds/ Total Number of Seeds) x 100 (1)

2.2 Influence of Cytokinin on Shoot Multiplication from Cotyledonary Nodes Explant

After 15 days- old seedling, the shoot tip and radical were excised and cotyledonary nodes (0.5-10 mm.) which were used as explant in all experiments. After that, explants were cultured in bottles containing around 25 ml from MS sold media supplemented with plant growth regulator (6-Benzylaminopurine) (BAP) at different concentrations from 0.0., 1.0, 3.0 & 5.0 mg/l. Cultured 40 bottles for all BAP concentrations, 3 explants in each bottle with 10 replicas for each concentration. All BAP treatments were transferred to growth chamber for four weeks under standard culture conditions the photoperiod light (12 hr/day), the fluorescent light intensity was 1000 Lux and temperature was maintained at 25±2°C.

After four weeks of culture, the following parameters were recorded:

Explants responding (%) = No. Of Adventitious Buds from Explants /Total No. of Explants) × 100 (2)

Number of Shoots = Total No. of Shoots for all Replica / No. of Replica (3)

2.3 Influence of Cytokinin-Auxin Combinations on Shoot Multiplication from Cotyledonary Nodes Explant

For multiple shoot induction, cotyledonary nodes explants were transferred to MS media contain different concentrations from BAP combined with different concentrations of α-naphthalene acetic acid (NAA). Data were recorded after four weeks from culture, so all cultures were kept under the standard culture conditions for photoperiod light (12hr/day), the fluorescent light intensity was 1000 Lux and temperature at 25±2^{o}C as mentioned above. After that, explants responding % and number of shoot calculated.

2.4 Influence of Sucrose Concentrations on Shoot Multiplication from Cotyledonary Nodes Explant

This experiment was conducted based on the results of previous experiments. The MS media contain 3mg/l of BAP supplemented was best treatment for produced number of shoots from Cotyledonary nodes explants. By using different concentration from sucrose (5, 10, 20, 30, 50 g/l) on MS media contain 3mg/l of BAP supplemented. Experiment was performed with 10 replicates and three explants per bottle were cultured. Data were recorded after four weeks from culture, so all cultures were kept under the standard culture conditions for photoperiod light (12hr/day), the fluorescent light intensity was 1000 Lux and temperature at 25°C ± 2 ^{o}C as mentioned above after that calculated explants responding (%) and number of shoot.

2.5 Rooting and Acclimatization

The shoots which obtained from the multiplication stage were transplanted to tubes contained liquid media (MS full strength, MS half-strength and MS half-strength medium supplemented with 0.2 mg/l NAA) for induce roots formation. Data Recorded for rooting percentage and number of roots after 4 weeks from culture. Collected Healthy plantlets from root induction medium and washed with sterile distilled water to remove all the adherent traces of media. Then, all the healthy plantlets transferred to Vermokliet soil, watered regularly and covered with plastic bags, for completing their acclimatization stage.

2.6 Statistical Analysis

Statistical analyses were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Quantitative data were described using mean and standard error. Significance of the obtained results was judged at the 5% level. The used tests were F-test (ANOVA) for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (*LSD*) for pairwise comparisons [15].

3. RESULTS AND DISCUSSION

3.1 Influence of Cytokinin on Shoot Multiplication

Overall, the data presented in Table 1. indicated that the Impact of BAP different treatments responded to Shoot Multiplication highly significantly ($P \leq 0.001$) and the response rate was about 73% for all BAP treatments compared with control (33.33% - 0.0 BAP). Similar results found and agree with the findings by Mukhtar et al. [16] who reported the shoot formation percentage increases in the presence of BAP growth regulator. However, similar results were also obtained by Hussain et al. [17] who reported that 1 mg/l of BAP was most effective in shoot induction percentage by 82% in nodal segments explants. These were results indicated that concentration of growth regulators (BAP) have significant effect on the shoot formation percentage, on the other hand, the MS media without any concentrations from BAP, stimulated shoots and roots formation for some explants. 6-Benzyl-aminopurine (BAP) is consider one of various types of cytokinins that can stimulate lateral buds growth and can cause multiple shoot formation by breaking shoot apical dominance [18]. Plant growth regulators are widely used to control the growth and development of tissue plants, relying on the species used, where in low concentrations affect plant growth and development by stimulating, inhibiting or altering the physiological processes in plant tissues, for that the plant growth regulators are requirement

for the success of tissue culture [19]. Cytokinins consider one of the important plant hormones to promote cell division and differentiation [20].

Moreover, results from the experiment showed that the influence of growth regulator (BAP) on number of shoots from cotyledonary nodes highly significantly (*P ≤ 0.001*) as shown in Table 1. Generally the number of shoots increased with increasing BAP concentrations whereas; the highest mean number of shoots (No. 3.40) observed by MS media 3 mg/l BAP compared with control. These results agree with obtained by Patil et al. [21], they found that the highest shoots numbers (7.10) were recorded in MS medium supplemented with BAP 3 mg/l. On the other hand, the results indicated that the number of shoots decreased in 5 mg/l supplemented with BAP and callus were appeared at the base of the shoots compared with control. These results agree with what founded by Weremczuk-Jeżyna et al. [22] where decreased to 2.9 shoots per explant were cultured on 5 mg/l for *D. forrestii*.

3.2 Influence of Cytokinin-Auxin Combinations on Shoot Multiplication

Overall the data presented in Table 2. indicated that the combinations between cytokinin-auxin (BAP–NAA) highly significantly (*P≤ 0.001*) and less effective on explants responding (%), whereas the high Explants Responding (%) was in media supplemented BAP without NAA compared other treatments. Furthermore, it was noted that the media with combination of BAP and NAA gives formation of callus in bases of the plantlets as shown in Fig. 2. When the concentration of NAA increased compared with BAP, the lowest Explants Responding (%) occurred as 6.67%. (1 mg/l BAP + 2 mg/l NAA). In most plant, the ratio of auxin and cytokinins is a determinant in meristem formation and type of shoot formation was stimulated by balance of hormones, and the ratio usually different according to genuses and species in terms of plant sensitivity to the hormones Razdan [23]. In tissue culture the auxins used in promoting the growth of plant roots and adventitious roots, Auxins as other hormones its different concentration also resulting in different results depending on plants species and genuses [24].

The data presented in Table 2. indicated that the combinations between cytokinin-auxin (BAP–NAA) highly significantly (*P≤ 0.001*) and less effective on number of shoots, whereas high number of shoots was in media supplemented BAP without NAA but the highest number of shoot was 3.40 in media with 3 mg/l BAP compared other treatments. George [25] mentioned that the BAP is commonly used within the concentration range of 1.0 – 3.0 mg/L for shoot multiplication because higher concentration of BAP can caused inhibited of shoot buds because excess hormone causes toxic to the plant. Moreover, the least number of shoot was (0.20, 0.60) that were showed in media contained (1 BAP+ 2 NAA mg/l, 5 BAP+ 0.5 NAA mg/l). Also, we noticed increased callus size in bases of plant lest with were raised NAA concentration. Saglam [26] showed that the medium supplement 0.5 mg/l of BAP without addition NAA showed well developed green leaves with maximum number shoots from cotyledon node explant of sainfoin plant, this agree with our results. In some plants increasing the cytokine concentrations in media gives a decrease in the rate of regeneration, but in a media without growth regulators the rate of regeneration increasing [27, 28]. Whereas, Seyyedyousefi et al. [29], they found that the medium supplemented with certain concentrations of BAP and NAA influenced on shoot multiplication of *Alstroemeria* plant. From the results for all found that the effect plant growth regulators depending on species and genuses of plant.

Fig. 2. BAP 3mg/1 + NAA N 0.5 mg/1

Table 1. Influence of 6-Benzylaminopurine (BAP) different Concentrations on Explants responding (%) and number of shoots (n = 10) for *Chenopodium Quinoa* Willd. Plant

BAP mg/l	Explants responding (%)	Number of shoot
0.0	$33.33^{b} \pm 7.03$	$1.40^{c} \pm 0.45$
1.0	$73.33^{a} \pm 8.32$	$2.80^{ab} \pm 0.33$
3.0	$73.33^{a} \pm 8.32$	$3.40^{a} \pm 0.34$
5.0	$73.34^{a} \pm 4.44$	$2.20^{bc} \pm 0.13$
F	7.715^{*}	6.570^{*}
p	$<0.001^{*}$	0.001^{*}
LSD 5%	20.654	0.9561

*Data was expressed using Mean ± SE. Means with Common letters are not significant (i.e. Means with Different letters are significant) *: Statistically LSD at p ≤ 0.05*

Table 2. Influence of combination between 6-benzylaminopurine (BAP) and α-naphthalene Acetic Acid (NAA) different concentrations on explants responding (%) and number of shoots (n = 10) for *Chenopodium quinoa* Willd. plant

BAP mg/l + NAA mg/l	Explants responding (%)	Number of shoot
1 mg/l + 0.0mg/l	$73.33^{a} \pm 8.32$	$2.80^{ab} \pm 0.33$
1 mg/l + 0.5 mg/l	$33.33^{de} \pm 7.03$	$1.0^{def} \pm 0.21$
1 mg/l + 1 mg/l	$40.0^{cd} \pm 8.32$	$1.40^{cde} \pm 0.34$
1 mg/l + 2 mg/l	$6.67^{f} \pm 4.44$	$0.20^{f} \pm 0.13$
3 mg/l + 0.0 mg/l	$73.33^{a} \pm 8.32$	$3.40^{a} \pm 0.34$
3mg/l + 0.5 mg/l	$66.67^{ab} \pm 7.03$	$3.0^{ab} \pm 0.37$
3 mg/l + 1 mg/l	$53.33^{bc} \pm 5.44$	$1.80^{cd} \pm 0.25$
3 mg/l + 2 mg/l	$60.0^{ab} \pm 8.32$	$1.80^{cd} \pm 0.25$
5 mg/l + 0.0mg/l	$73.34^{a} \pm 4.44$	$2.20^{bc} \pm 0.13$
5 mg/l + 0.5 mg/l	$20.0^{ef} \pm 5.44$	$0.60^{ef} \pm 0.16$
5 mg/l + 1 mg/l	$53.33^{bc} \pm 5.44$	$2.20^{bc} \pm 0.25$
5 mg/l + 2 mg/l	$66.67^{ab} \pm 9.94$	$2.80^{ab} \pm 0.49$
F	9.838^{*}	11.846^{*}
P	$<0.001^{*}$	$<0.001^{*}$
LSD 5%	19.863	0.811

*Data was expressed using Mean ± SE. Means with Common letters are not significant (i.e. Means with Different letters are significant) *: Statistically significant at p ≤ 0.05*

3.3 Influence of Sucrose Concentrations on Shoot Multiplication

Overall, the data presented in Table 3 . found that the highly significantly (*P≤ 0.001*) between sucrose treatments and explants responding (%). The highest of explants responding (%) 93.33% was in media containing (sucrose 10 g/l + 0.0 BAP) and (sucrose 10 g/l + 3 mg/l BAP). While the lowest explants responding (%) was 53.33% with sucrose both concentrations (Sucrose 5 g/l + 3g/l BAP and Sucrose 50 g/l + 3 g/l BAP). On the other hand, the supra-optimal sugar level for good greening and developed shoots (4 shoots) was in medium supplement with sucrose 10 g/l without BAP. Number and greening colour of shoots decreased with increasing sucrose concentration, the green colour disappeared completely with sucrose 50 g/l + 3g/l BAP compared with control as showed in Figs. $3_{A, B \& C}$. The our results agree with the results obtained by Burnouf-Radosevich and Paupardin [30] the beneficial effect of low sucrose level on greening and vitality of quinoa shoots suggest that a supra-optimal sugar (sucrose) concentrations may interfere with photosynthesis of tissues cultured in vitro as in spinach and other species. Also, they have been demonstrated the negative effect of high and lower levels of sucrose sugar on greening and developing of shoots.

In plant tissue culture medium, often used sucrose as a carbon source to provide energy for morphogenesis and adventitious shoot regeneration from explants [31]. Moreover, Sucrose induce osmotic stress and it causes regulate metabolic processes like abscisic acid synthesis, auxin transport and starch accumulation in plant cell and tissues, hence

enhanced or reduced the growth and development of explants [32, 33]. Regarding quinoa, it has been found that sucrose concentration may play a role of developing and greening of shoots. From the above experiences, we noticed that the color of greening decreased from shoots, consequently the effect of different sucrose treatments was studied (5, 10, 20, 30, 50 g/l).

3.4 Rooting and Acclimatization

Overall the data presented in Table 4. showed that the highly significantly (P≤ 0.001) between all MS media strength and roots percentage % also between number of roots. The highest roots responding 60% was in half strength of MS media that outperformed also in number of roots (0.8) compared with control (MS). On the other hand, the root formation was not observed when shoots were cultured on full strength media (MS) as shown in Fig. 4. For acclimatization, the rooted shoots were removed from tubes and washed thoroughly to remove remnants of media and transplanted to small pots containing potting soil. Plants were covered to ensure high humidity as shown in Fig. 5. Thereafter, the plantlets were transferred to green house to follow their development and produced the 2nd Generation (F2). Good effect of half-strength medium for rooting is in conformity with the results recorded for many plant as Lavendula *latifolia* [34], Lavendula dentata [35], Prunella Vulgaris [36] and Vanda pumila [37].

Table 3. Influence of 6-benzylaminopurine (3 mg/l - BAP) with different concentrations of sucrose on explants responding (%) and number of shoots (n = 10) for *Chenopodium quinoa* Willd. Plant

Sucrose (g/l) + 3mg/l BAP	Explants responding (%)	Number of shoot
10g/l + 0.0 BAP	$93.33^{a} \pm 4.44$	$4.0^{a} \pm 0.0$
5g/l + 3mg/l BAP	$53.33^{b} \pm 11.33$	$1.80^{b} \pm 0.44$
10g/l + 3mg/l BAP	$93.33^{a} \pm 4.44$	$4.20^{a} \pm 0.53$
20g/l + 3mg/l BAP	$60.0^{b} \pm 10.89$	$3.40^{a} \pm 0.81$
30g/l + 3mg/l BAP	$73.33^{ab} \pm 8.32$	$3.40^{a} \pm 0.34$
50g/l + 3mg/l BAP	$53.33^{b} \pm 11.33$	$1.80^{b} \pm 0.33$
F	4.335^{*}	4.956^{*}
p	0.002^{*}	0.001^{*}
LSD 5%	25.464	1.3455

*Data was expressed using Mean ± SE. Means with Common letters are not significant (i.e. Means with Different letters are significant) *: Statistically significant at p ≤ 0.05*

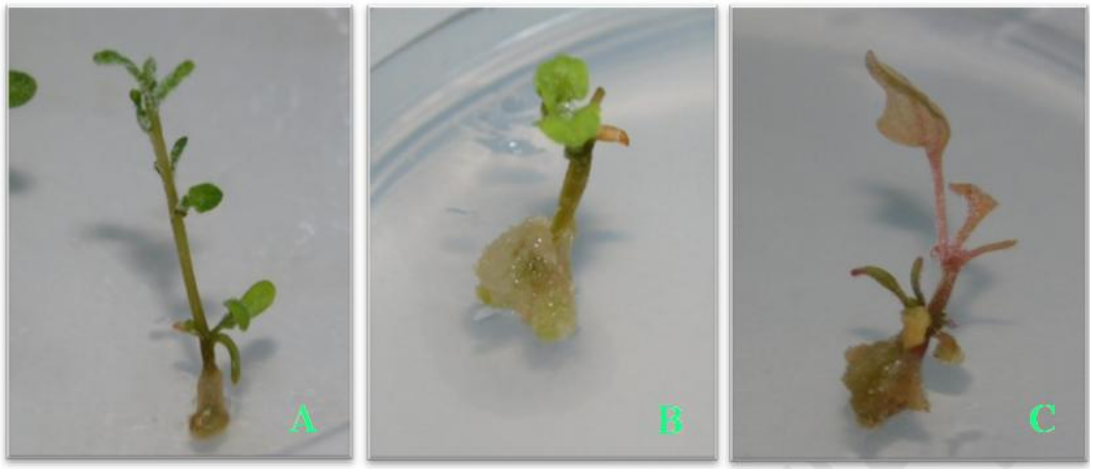

Fig. 3(A,B&C). Influence of different sucrose concentrations on developed and greening of *Chenopodium quinoa* willd. plant, (A) Sucrose 10 mg/l +0.0 BAP, (B) Sucrose 5 mg/l + 3mg/l BAP, (C) Sucrose 50 mg/l + 3 mg/l BAP

Table 4. Influence of different strength MS in the presence or absence of NAA on root percentage (%) and number of roots (n = 10) for *Chenopodium quinoa* willd. plant

MS media	Root percentage (%)	Number of root
MS	0.0^{b} ± 0.0	0.0^{b} ± 0.0
½ MS	60.0^{a} ± 16.33	0.80^{a} ± 0.25
½ MS + 0.2 mg/l NAA	20.0^{b} ± 13.33	0.40^{ab} ± 0.27
F	6.300*	3.600*
p	0.006*	0.041*
LSD 5%	35.319	0.6117

*Data was expressed using Mean ± SE. Means with Common letters are not significant (i.e. Means with Different letters are significant) *: Statistically significant at p ≤ 0.05*

The superiority of the ½MS medium may be attributed to that the reduction in the levels of salts in the medium means a reduction in the level of nitrogen in medium, which leads to a reduction in its internal level in the shoots, this consequently may lead to an increase in the proportion of carbohydrates to nitrogen, which leads to increase the formation of roots [38, 39]. It should be noted that, used NAA with half strength of MS caused callus formation in bases plantlets and defoliation, which may be attributed to production of ethylene induced by the auxine (NAA) [30].

Fig. 4. Formation of shoot and Root on ½ MS liquid for *Chenopodium quinoa* willd. Plant

Fig. 5. Acclimatization of *Chenopodium quinoa* willd. Plant

4. CONCLUSION

The results present form this study, indicated that the concentration of sucrose (10 g/l) without Plant growth regulators (BAP & NAA) or with 3mg/l BAP gives more effective on the proliferation of large number and good greening of quinoa shoots plant. A good percentage of roots were developed on ½ MS medium. Hence the present result could be useful protocol for the propagation quinoa species, but, the rooting stage needs more studies for increasing rooting number which are important for acclimated quinoa plant to development regeneration. So, we have developed the regeneration protocol for quinoa plant by using media containing 3 mg./l BAP and 10g/l sucrose without NAA, this protocol has a sufficiently high success rates to be the starting point for development of quinoa plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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