



Assessment Various Concentrations of ZnO-Nanoparticles on Micropropagation for *Chenopodium quinoa* Willd. Plant

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Authors' contributions

This work was carried out in collaboration between both authors. Authors FRAG and HEAE 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

Assessment influences various concentrations of ZnO-Nanoparticles (ZnO-NPs) on *Chenopodium quinoa* Willd. Micro-propagation by cotyledonary nodes explants was achieved. We used different concentrations of ZnO-NPs (0.2, 2, 10, 20 mg/l) and used the medium free from ZnO-NPs as control. The results indicated that the presence ZnO-NPs in medium was good effect on germination rate of quinoa seeds at 2 mg/l concentration, and we noticed a density in roots hair and number or length roots of seedling. In addition, the highest responding of explant to ZnO-NPs 93.33, 80.8% were in MS that supplemented 0.0, 2.0 mg/l respectively. Maximum numbers of roots 4.0 were also observed in MS containing 0.0, 2.0 mg/l. Although, there was positive clearly effect on number leaves of shoots, but there was a sudden decline (from 8.43 to 1.0) occurred by increasing ZnO-NPs concentrations from 2.0 to 20.0 mg/L. However, the ZnO-NPs do not effect on length of shoots, where the lengthiest shoot occurred in MS without ZnO-NPs. Regarding rooting shoots, there was significant effect of ZnO-NPs on both percentage of root and root number, where percentage of root reached to 100% in 10.0 mg/l concentration and roots number was 4.80 roots in the same concentration.

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ABBREVIATIONS

NPs (Nanoparticles); ZnO-NPs (ZnO Nanoparticles); NaOCl (Sodium Hypochlorite); MS (Murashige and Skoog medium). GR (Germination rates); ROS (reactive oxygen species).

1. INTRODUCTION

Agriculture forms the backbone of the economy of most countries and is considered the fundamental contributor to their overall growth, industrialization, and modernization. Agriculture has made remarkable advances over the past decades, but climate change, increasing global food demand and agrochemicals demand novel and sustainable agricultural practices to improve crop yield and quality. Various strategies using nanotechnology have been explored widely to provide solutions [1-2]. Nanotechnology, a new emerging and fascinating field of science, and in recent year's remarkable progress has been made in developing nanotechnology. The growth of nanotechnology has led to the rapid development of commercial application which involves the use of a great variety of manufactured nanoparticles and which have ability to revolutionize the agriculture and food industry. Thereby, there is a crucial urgency to perform further studies on the use of nanoparticles in the agricultural field [3-7].

The term nanotechnology comes from the combination of two words: the Greek numerical prefix nano referring to a billionth and the word technology. As an outcome, Nanotechnology or Nano-scaled Technology is generally considered to be at a size below 100 nm (a nanometer is one billionth of a meter, 10^{-9} m). Nanoscale science (or nanoscience) studies the phenomena, properties, and responses of materials at atomic, molecular, and macromolecular scales, and in general at sizes between 1 and 100 nm. In this scale, and especially below 5nm, the properties of matter different significantly from that at a larger particulate scale [8-9]. On the other hand, Nanoparticles (NPs) that ranging in size from 1 to 100 nm possess specific physico-chemical properties attributed to smaller size, large surface area and high reactivity compared to their bulk counterparts [10].

The path of nanoparticles synthesis and their relative size and structure plays pivotal role in exhibiting the biological properties of nanoparticles [11]. The interaction of nanoparticles with the biological system is of large importance, and nowadays researchers are trying to find the potential effects of various kinds of nanoparticles in plants, animals and humans [12]. Therefore, NPs can serve as "magic bullets", containing herbicides, Nano-pesticide, fertilizers, or genes, which target specific cellular organelles in plant to release their content [13].

Zinc oxide (ZnO) one of the most used Nano-products, are used in food packaging and drugs due to their superior antimicrobial efficacy [14-15]. ZnO-NPs are also used in sun-protective lotions, wall paints, ceramic manufactures, or sporting goods [16-17]. ZnO-NPs plays active role in regulating various mechanisms involved in response to abiotic stresses in plants. It has been found that zinc has an important role in the management of reactive oxygen species (ROS) and protection of plant cells against oxidative stresses [18]. According to [19], ZnO-Nanoparticles considered environment-friendly and hence widely used in biological applications.

The plant tissue culture has a great impact on both agriculture and industry, through providing plants needed to meet the ever increasing world demand. It has made significant contributions to the advancement of agricultural sciences in recent times and today they constitute an indispensable tool in modern agriculture [20]. Plant tissue culture has contributed enormously to agricultural biotechnology. For example, many plants grown from seed show considerable variation in growth, flower characteristics, yield, disease resistance, resistance to environmental stress, and so forth [21-22]. It would therefore be valuable to select those that possess desirable characters for vegetative multiplication [23].

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 (APG III) ranks quinoa in the family Amaranthaceae [24]. 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 [25]. 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 [26].

According to our knowledge, the effect of ZnO Nano-particles on different stages of quinoa micro propagation, such as shoot multiplication, shoot elongation, and rooting formation as well as their phytotoxicity is not well documented. Consequently, the main aim of the present study is to observe the potential effects of ZnO-Nanoparticles on developed quinoa micro-propagation.

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 its multiple times with sterilized distilled water, (this was done under a laminar flow hood), the culture media which used in all experiments were MS (Murashige and Skoog medium) [27]. 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.

2.2 Preparation of ZnO-NP Suspension

For ZnO-Nanoparticles suspension preparing, we used M. Helaly et al. [28] method with some modified as follow: ZnO-Nanoparticles was filtered by 0.22 µm glass filter without using centrifuge machine. ZnO-Nanoparticles (average particle size 35-45 nm) were purchased from US Research Nano-materials, Ink Company, USA. 0.15 g of solid ZnO-NPs was dissolved in 100 ml distilled water, and a magnetic stirrer was used to homogenize the solution at 40 kHz for 30 min. For avoid aggregation of the particles, small magnetic stirrer bar was placed in the suspensions which was automatically stirred thoroughly before use. Then, ZnO-Nanoparticle suspensions was filtered by (0.22 µm glass filter) prior to being added to culture media. Different concentrations of ZnO-NPs (0.2, 2, 10, 20 mg prepared and the media without ZnO nanoparticles used as control [28].

2.3 In vitro: Seed Germination

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 (12hr/ 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:

$$\text{Germination rate \%} = \frac{\text{Total Number of Germinated Seeds}}{\text{Total Number of Seeds}} \times 100 \quad (1)$$

2.4 Plant Regeneration

After 15 days- old seedling, (which grown in ZnO-NPs medium) 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 (free ZnO-NPs) with adding 10 g/l sucrose and solidified with 6 g/l agar. All treatments were transferred to growth chamber for 4 weeks under standard culture conditions the photoperiod light (12hr/day), the fluorescent light intensity was 1000 Lux and temperature was maintained at 25±2°C. After 4 weeks of culture, the following parameters were recorded:

$$\text{Explants responding \%} = \frac{\text{No. of Adventitious Buds from Explants}}{\text{Total No. of Explants}} \times 100 \quad (2)$$

$$\text{Number of Shoots (No.)} = \frac{\text{Total No. of Shoots for all Replica}}{\text{No. of Replica}} \quad (3)$$

$$\text{Shoot Length (cm)} = \frac{\text{Total length of shoots for all Replica}}{\text{No. of Replica}} \quad (4)$$

$$\text{Number of leaves (No.)} = \frac{\text{Total No. of leaves for all Replica}}{\text{No. of Replica}} \quad (5)$$

2.5 Rooting Formation and Acclimatization

The shoots which obtained from the multiplication stage were transplanted to tubes contained liquid media contained half-strength medium (½ MS) 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. After two weeks around, the covers were removed gradually to harden the plantlets. Then, the acclimatized plants were shifted to a greenhouse for completing their developed.

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 [29].

3. RESULTS AND DISCUSSION

3.1 Influence of ZnO-Nanoparticles on Germination Rate (%)

The results of current study showed that the role of ZnO Nanoparticles concentrations (0.2, 2.0, 10.0 & 20.0 mg./l) played an increasing in the germination rate of quinoa plant up to a certain level (2.0 mg/l ZnO nanoparticles), but with increased ZnO Nanoparticles concentrations maybe cause toxicity if the ZnO-NPs concentration more than 20.0 mg/l as shown in Fig. 1. The highest germination rate (22.0%) was

in MS medium supplemented with 2.0 mg/l of ZnO Nanoparticles, while, the lowest germination rate (12.0%) occurred in MS medium containing 20.0 mg/l of ZnO-Nanoparticles. This means that increasing concentration ZnO-NP in medium could be led to toxicity for germination of quinoa seeds, this agrees with what found by Zafar et.al. [30] they found the highest concentrations (500 to 1500 mg/l) of ZnO-NP in medium adversely affects seed germination and seedling growth of *Brassica nigra* and also lead to an increase in the antioxidative activities and non-enzymatic antioxidants. ZnO-Nanoparticles improve the germination and seedling vigor of a wide of crops such as tomato [31], *Cucumis sativus* [32], groundnut [33].

Senthilkumar and Sridhar [31-34] they observed that the metal oxide nano-particles are efficient enough to improve the germination of aged seeds of black gram and tomato up to 30 percent. This may be due to overcome of reactive oxygen species (ROS) generated during seed storage. Both positive and negative effects was showed when some higher plants were treats with of several Nano Particles TiO₂, ZnO, Mg, Al, Pd, Cu, Si, C60 fullerenes, and multiwall carbon nanotubes [7].

On the other hand, we noticed density in roots hair and number or length roots of seedling, also, increasing of shoot length in medium supplemented with 0.2, 2, 10 mg/l of Zn-NPs Fig. 2. The our results agree with the results obtained by Raja et al. [35] they that recorded maximum germination, root length, shoot length and seedling vigor in seed that treats by Zn-NPs when compared to untreated seeds.

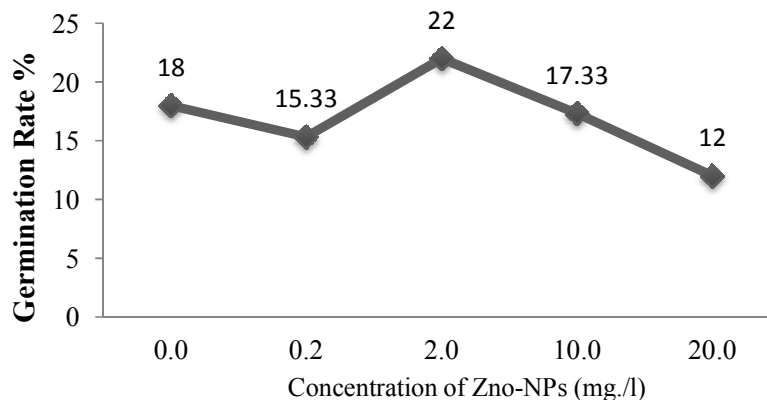


Fig. 1. The influence of ZnO-NPs (0.0, 0.2, 2.0, 10.0 & 20.0 mg/l) concentrations on seeds germination rates (%)

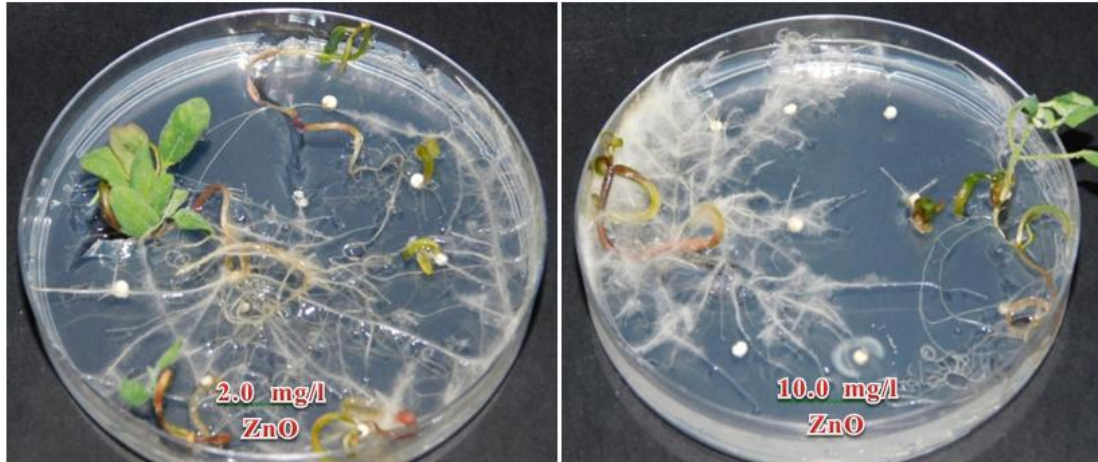


Fig. 2. The influence of ZnO-NPs (2.0 mg/l and 10.0 mg./l) concentrations on seeds germination rates (GR %)

3.2 Effect ZnO Nanoparticles on Growth Parameters

Overall the data presented in Table 1 indicated that the responding explant (%) to ZnO-NPs concentrations was highly significantly ($P \leq 0.001$), maximum explant responding % 93.33, 80.8 were in 0.0 and 2.0 mg./l ZnO-NPs MS medium respectively, while the minimum responding explant (% 6.67) was obtained from explant that treated with 20.0 mg/l ZnO-NPs. Regarding shoot number, the data presented in Table 2 indicated that the difference between all ZnO-NPs treatments was highly significantly ($P \leq 0.001$), where, the highest number of shoot (4.0) occurred in MS medium containing 0.0 & 2.0 ZnO-NPs concentrations. Then, the shoot numbers began to decrease with increasing ZnO-NPs concentration until it reached the lowest number (0.20) at 20.0 mg/l of ZnO-NPs Fig. 3.

The results of current study could agree with [36] they found that the highest percentage of shoot regeneration (89.6%) was when nodal explants of *Stevia rebaudiana* were cultured on callus induction medium treated with 1 mg/l ZnO-NPs. On the other hand, [37,38] found that low concentrations 10 mg/l, it stimulated the callus growth and indicated the nanoparticles role in decontamination, regeneration, organogenesis, callus formation and activated a protein that had a vital role in growth. However, that callus biomass decreased with increasing of ZnO-NPs concentration, but with increasing concentration of ZnO-NPs in medium, the responding of explant decreased. Also, Callus proliferation was

observed in all explants with all of the ZnO NPs treatments, but it reduces in high ZnO NPs concentration [39].

On the other hand, from the Table 1 we noticed that there are difference significantly between treatments on shoot length, but do not effect ZnO-NPs on shoot length, where the longest shoot 2.33 cm was in MS medium (0.0 mg/l ZnO-NPs), while the shortest shoot (0.10 cm) was in medium that containing 20.0 mg/l ZnO-NPs. In addition, although there was positive clearly effect on number of leaves but there was a sudden decline in numbers (8.43-1.0) occurred by increasing ZnO-NPs concentration from (2.0 - 20 mg/L).

In generally, the mechanism that causes increase of growth with increasing concentration nanoparticles then a drop occurs in the growth and metabolism after reaching a certain threshold might involve the abundance of reactive oxygen species (ROS) and free radicals of Zn^{+1} ions. This ROS and free radicals produced by ZnO-NPs are internalized into the plant cell wall, cell membrane, cytoplasm, and nucleus. The translocation of ROS into the plant cells cause their destruction that occurs by the degradation of DNA and mitochondrial membranes [40-42]. Also, the denaturation of proteins and peroxidation lipids occur of cell wall leading to mutagenesis. Consequently, the inflammatory signaling cascades are activated, causing genotoxicity. Hence, the growth parameters and secondary metabolites show a fall [43].

Table 1. Impact of different concentration of ZnO-NPs on percentage of shoot responding %, number of shoots, shoots length (cm), number of leaves (n=10)

ZNO-NPs conc.	Shoot responding %	Shoots number (No.)	Shoot length (cm)	Leaves number (No.)
0	93.33 ^a ± 4.44	4.0 ^a ± 0.0	2.33 ^a ± 0.53	6.75 ^a ± 1.28
0.2 mg	53.33 ^c ± 5.44	2.20 ^b ± 0.25	1.55 ^a ± 0.40	7.93 ^a ± 0.89
2 mg	80.0 ^{ab} ± 5.44	4.0 ^a ± 0.60	1.63 ^a ± 0.17	8.43 ^a ± 0.46
10 mg	60.0 ^{bc} ± 12.96	2.0 ^b ± 0.42	1.57 ^a ± 0.41	8.33 ^a ± 1.53
20 mg	6.67 ^d ± 4.44	0.20 ^c ± 0.13	0.10 ^b ± 0.07	1.0 ^b ± 0.67
F	20.584 [*]	20.641 [*]	5.145 [*]	9.085 [*]
p	<0.001 [*]	<0.001 [*]	0.002 [*]	<0.001 [*]
LSD 5%	20.802	0.9976	1.023	2.967

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

3.3 Rooting Formation and Acclimatization

Overall, the data presented in Table 2 showed that the highly significantly (P≤ 0.001) between all ZnO-NPs treatments and rooting formation and root numbers, whereas a positive effect of ZnO-NPs was most clearly on root formation (%) and number of root in both concentration (2.0 & 10.0 mg/l ZNO-NPs), while the other both concentration (0.2, 20.0 mg/l. ZnO-NPs). The best concentrations for root formation (100, 80 %) were at 10, 2 mg/l. ZnO-NPs respectively. In addition, the maximum number of root (~ 5) was in explants that treated with 10.0 mg/l of ZnO-NPs, while no rooting formation took place in explants that treated with 20.0 mg/l of ZnO-NPs.

According to [44-47] they reported that can be explained induction of roots in two ways: (i) function of zinc in biochemical process, and (ii) role of ROS. The acidic nature of MS medium could increase dissolution of ZnO-NPs into zinc ion. Zinc ion may play role as cofactor for

several enzymes such as oxidases, dehydrogenases, a hydrases, and peroxidases, in regulating auxin synthesis, nitrogen metabolism, cell multiplication. Zinc itself regulated the synthesis of endogenous plant hormones; also presence auxin regulates local synthesis of cytokinin by controlling the expression of adenosine phosphate–isopentenyltransferase (PsiPT) gene, which encodes a key enzyme in cytokinin biosynthesis.

Zinc itself regulated the synthesis of endogenous plant hormones, also presence auxin regulates local synthesis of cytokinin by controlling the expression of adenosine phosphate–isopentenyl transferase (PsiPT) gene, which encodes a key enzyme in cytokinin biosynthesis [48]. Furthermore, ROS generated in explants works as signaling molecule at non-toxic level, where it involved in induction of roots [49]. However, another aspect of ROS growth regulation involves superoxide, that affects root growth and root hair development through the regulation of calcium channels [50-51].

Table 2. Influence of ZnO-NPs different concentration on roots formation (%) and number of root (n = 10)

ZNO-NPs Conc.	Root Formation (%)	Root number (No.)
0	60.0 ^b ± 16.33	0.80 ^{bc} ± 0.25
0.2 mg	20.0 ^c ± 13.33	1.0 ^{bc} ± 0.52
2 mg	80.0 ^{ab} ± 13.33	1.60 ^b ± 0.27
10 mg	100.0 ^a ± 0.0	4.80 ^a ± 0.74
20 mg	0.0 ^c ± 0.0	0.0 ^c ± 0.0
F	13.821 [*]	18.126 [*]
p	<0.001 [*]	<0.001 [*]
LSD 5%	31.775	1.242

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

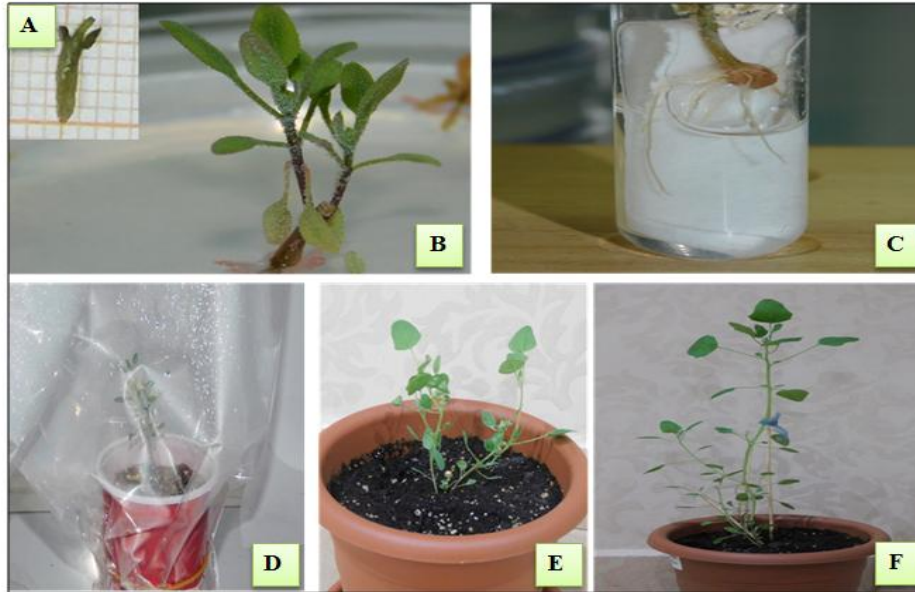


Fig. 3. Micropropagation stages of quinoa plant under influence of zno-pns different concentrations (A) cotyledonary node explant (B) multiplication shoots (C) rooting shoots stage (D) acclimatization stage (E) & (F) quinoa plant at green house

In contrast, the toxicity of NPs determined by concentration of NPs and plant species, the studies that reported on phytotoxicity of ZnO-NP on plants are limited. Some plant species are sensitive toward ZnO-NP as rape, corn, lettuce, radish, ryegrass, cucumber [52], zucchini [53], garden cress, and broad bean [54], and wheat [55] are sensitive toward ZnO NP. Presence ZnO-NPs surrounding environment of plant effected on physiology and biochemistry of plant. The toxicity of ZnO-NPs due to accumulation it in root tissue and root surface and dissolution of zinc ions from NPs along with other physiochemical properties [56-57].

For acclimatization, the rooted shoots were removed from tubes and washed thoroughly to remove remnants of media and transplanted to small pots containing Vermokliet soil. Plants were covered to ensure high humidity. Thereafter, the plantlets were transferred to green house to follow their development as shown in Fig. 3.

4. CONCLUSION

The results present from this study, concluded that the ZnO Nanoparticles could be positive effect on micropropagation of quinoa plant through increasing germination rate, number of shoot and leaves it. When increased ZnO-NPs concentrations in medium up to 20 mg/l, the

ZnO-NPs tends toward prevent plant growth. Regarding rooting formation stage, the positively effect of ZnO-NPs was clearly on both percentage and number of roots, where the percentage of roots reached to 100% at 10 mg/l ZnO-NPs concentration. Based on experimental, results we believe the concentrations of ZnO-NPs in range (2-10 mg/l) may a positive effect on micropropagation of quinoa plant especially on rooting formation stage, but the concentrations of ZnO-NPs that higher than 10 mg/l had a negative effect on growth and developed quinoa plant, while increased ZnO-Nanoparticles concentrations more than 20.0 mg/l. maybe cause toxicity on growth and development of plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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