Int. J. Nanosci. Nanotechnol., Vol. 14, No. 2, June. 2018, pp. 133-141

Application of ZnO Nanoparticles for Inducing Callus in Tissue Culture of Rapeseed

Seyed Mousa Mousavi Kouhi[1,*] and Mehrdad Lahouti[2]

[1]Department of Biology, Faculty of Science, University of Birjand, Birjand, Iran.
[2]Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran.

(*) Corresponding author: smmousavi@birjand.ac.ir
(*Received: 28 January 2017 and Accepted: 15 April 2017*)

Abstract

Present study has comparatively investigated the effect of zinc oxide nanoparticles (ZnO NPs), ZnO bulk particles (BPs), and relevant metal ions (Zn^{2+}) on the tissue culture of rapeseed (Brassica napus L.) to properly evaluate the impact of ZnO NPs on callus induction. For finding the best hormonal combination for inducing callus, hypocotyl explants were cultured on Murashige and Skoog (MS) media containing different combinations of plant growth regulators. After that, hypocotyl explants were cultured in MS media containing the best hormonal combination, supplemented with four different concentrations (10, 25, 50, 100 mg L^{-1}) of ZnO NPs, ZnO BPs, or equivalent concentrations of Zn^{2+}. Results showed that 10 mg L^{-1} ZnO NPs can induce callus and shoot regeneration, while the same concentrations of other treatments cannot. Callus growth was significantly retarded by 25, 50 and 100 mg L^{-1} of all three types of treatment in a dose-dependent manner. Under highest concentration of ZnO NPs, callus was not induced. It can be concluded that optimum concentration of ZnO NPs can be beneficial for inducing callus and/or shoot regeneration in the plant tissue culture.

Keywords: *Brassica napus, Callus, Shoot regeneration, ZnO nanoparticles.*

1. INRODUCTION

Main characteristic features of nanoparticles (NPs), the building blocks of nanotechnology, are high surface/volume ratio and high reactivity. These features differentiate NPs from bulk materials [1]. The critical size of the particles to show these unique features are smaller than 100 nm [2], the size that limits NPs definition. Zinc oxide (ZnO) NPs are among the most applicable NPs in many fields, such as consumer products [3, 4].

The effects of NPs on different features of plants are depended on NPs type, organism species, exposure period, and NPs dose. There are some studies that have reported ZnO NPs can be beneficial for seed germination or other aspects of plant growth [5]. In contrast, most studies, mainly used high concentrations of ZnO NPs, have reported their phytotoxicity [4, 6-17). However, Mousavi Kouhi et al. [18-20] have comparatively investigated the effects of ZnO NPs, their BPs, and Zn^{2+} on rapeseed and concluded that phytoxicity of NPs is not more than that of BPs or Zn^{2+}.

In recent years, nanotechnology has had novel applications in plant biotechnology and agriculture [1, 21]. However, investigation of the effects of NPs on plant tissue culture is scarce. Some studies in this field have reported that nanoparticles can be synthesized by plant callus [22-25]. Others have used NPs in plant in vitro propagation for biological decontamination [26-29].

However, present study has comparatively investigated the effect of different concentrations of ZnO NPs, ZnO BPs, and Zn^{2+} on tissue culture of rapeseed (*Brassica napus* L.), aiming to find the potential role of ZnO NPs, having unique features, in inducing or inhibiting callus and/or shoot regeneration. Moreover, our comparative study allows to distinguish NPs-specific or substance dependence of ZnO NPs.

2. MATERIALS AND METHODS

2.1. ZnO NPs and ZnO BPs

ZnO NPs powder with a size lower than 50 nm, and ZnO BPs were purchased from Sigma-Aldrich Corporation. Particle size and morphology of ZnO NPs were characterized by transmission electron microscopy (TEM, Model 912 AB, LEO, UK). Hydrodynamic diameters of ZnO NPs and BPs were analyzed by dynamic light scattering (DLS) that is described in our previous work [18].

2.2. Seedlings Growth Condition

Seeds of *B. napus*, cultivar Hayola 401 were firstly surface sterilized with 70% (v/v) ethanol (1 min) and then with 5.5% (v/v) sodium hypochlorite (10 min). Seeds were then rinsed three times with sterile deionized water and placed in jars (10 seeds per jar) containing 50% Murashige and Skoog (MS) [30] medium supplemented with 1% sucrose, without growth regulators and vitamins, and solidified with 0.8% agar. Jars were maintained at 24 ± 2 °C with 16/8 h photoperiod under fluorescent tube lights for one week. Hypocotyls of sterile seedlings were then used as a source of explants for additional studies.

2.3. Finding the Best Hormonal Combination for Callugenesis

For finding the best hormonal combination for inducing callus, hypocotyl segments (0.5-1 cm) were cultured in the jars containing MS media (3% sucrose, 0.8% agar, pH 5.8) with different combinations of plant growth regulators (Table), at 24 ± 2 °C with 16/8 h photo-period.

After six weeks, Fresh (FWC) and Dry weight of calli (DWC) were determined and callugenesis percentage (CP) [(number of explants containing callus/total number of explants) × 100] was calculated. Finally, the best hormonal combination for callugenesis was selected after data analyzing.

Table 1. *Different combinations of plant growth regulators for inducing callus abbreviated for addressing in text. 2,4-D: 2,4-dichlorophenoxyacetic acid; BA: Benzyladenine; NAA: Naphthaleneacetic acid.*

Hormonal combination	Abbreviation
Without plant growth regulators	C (Control)
1 mg L^{-1} 2,4-D	1 D
2 mg L^{-1} 2,4-D	2 D
1 mg L^{-1} 2,4-D + 1 mg L^{-1} Kinetin	D, K
1 mg L^{-1} NAA + 1 mg L^{-1} Kinetin	N, K
1 mg L^{-1} 2,4-D + 1 mg L^{-1} BA	D, B
1 mg L^{-1} NAA + 1 mg L^{-1} BA	N, B
1 mg L^{-1} BA	1 B
2 mg L^{-1} BA	2 B
1 mg L^{-1} NAA + 2 mg L^{-1} BA	1 N, 2 B
1 mg L^{-1} NAA + 3 mg L^{-1} BA	1 N, 3 B
1 mg L^{-1} NAA + 4 mg L^{-1} BA	1 N, 4 B

2.4. Investigation of the Callus Induction under Treatments

Four concentrations of ZnO NPs and ZnO BPs (10, 25, 50, 100 mg L^{-1}) were prepared by MS medium containing the best hormonal combination for inducing callus, 3% sucrose, 0.8% agar, and pH 5.8. Before solidifying agar, ZnO NPs and ZnO BPs suspensions were dispersed by an ultrasonic bath (Branson Ultrasonic Bath - Model 8510) for 45 min. Four concentrations of Zn^{2+} at equivalent molar concentrations i. e., 8, 20.1, 40.2, 80.3 mg L^{-1}, were also prepared by the same medium after dissolving calculated amounts of $ZnSO_4.7H_2O$. Hypocotyl segments (0.5-1 cm) were then cultured in the jars containing those media, maintained at 24 ± 2 °C with 16/8 h photoperiod. After six weeks, FWC, DWC and CP were determined, and the following traits were also calculated:

Relative water content (RWC) of callus = [(FWC – DWC) / FWC] × 100

Shoot regeneration percentage (SRP) = [number of regenerated explants/total number of explants] × 100

Although hormonal combination was not optimized for shoot regeneration,

however, since shoot regeneration was observed under some treatments after six weeks, SRP was also calculated.

2.5. Statistical Analysis

Each experiment had four independent replicates. Experiments were arranged in a completely randomized design. A one-way ANOVA test followed by Duncan's multiple range test using direct, arcsin-transformed, or square root transformed data was performed with MSTAT-C software ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

After analyzing CP (Figure 1a), FWC (Figure 1b) and DWC (Figure 1c) of the calli induced by different combinations of plant growth regulators, it was found that the medium containing 1 N, 2 B was the best one for callus induction. Thus, it was selected for investigating comparative effects of ZnO NPs, ZnO BPs and Zn^{2+} on callus induction. Other combinations of N, B also induced a high degree of callus induction with a high FWC and DWC (Figure 1 and 2) so that some of their calli showed shoot regeneration (Figure 2). However, the results showed that a 1:1 ratio of N, B was not efficient for induction of large calli, compared with their other combinations. In contrast, Akasaka-Kennedy and Yoshida [31] have used 1:1 ratio of N, B for callus induction of leaf explants of *B. napus*.

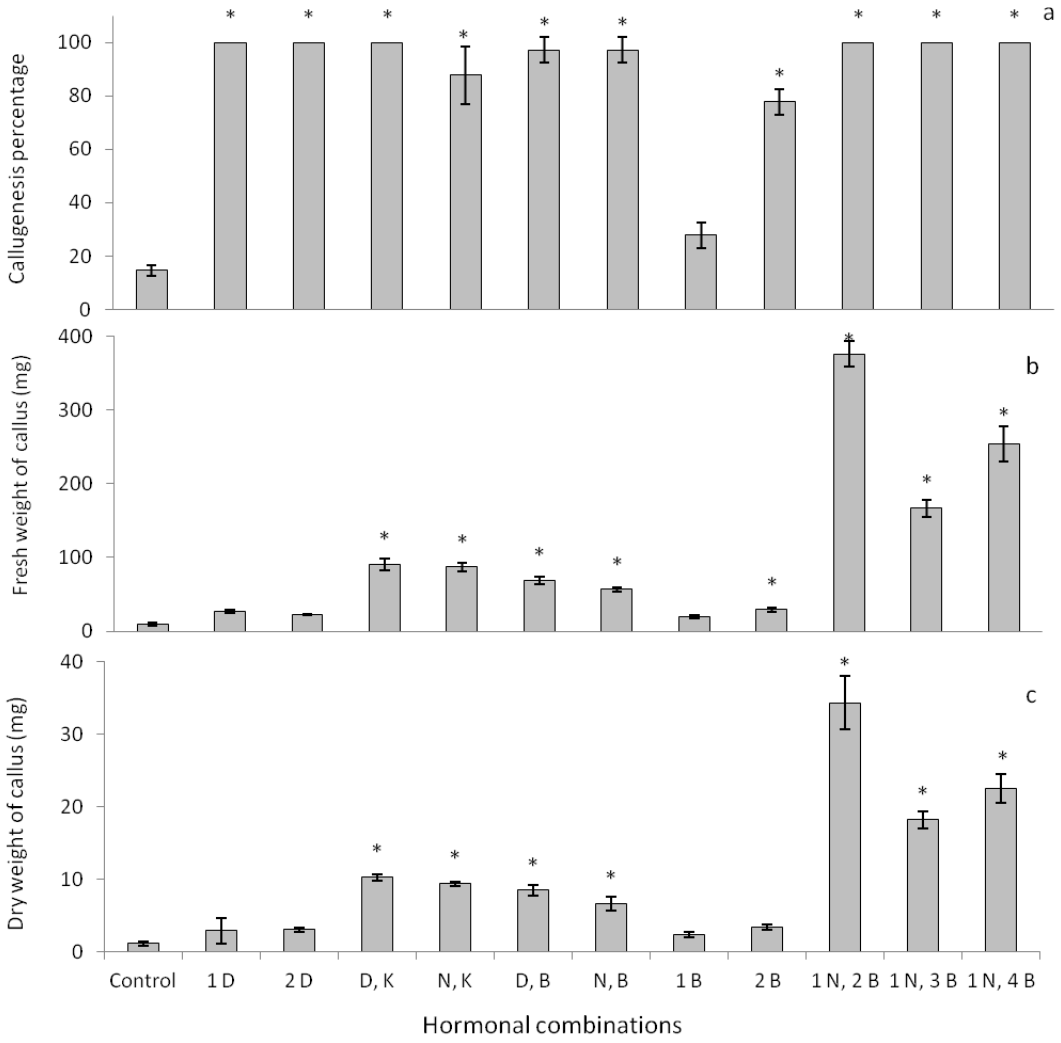

Figure 1. *Effect of different hormonal combinations on callugenesis percentage (a), Fresh (b) and Dry (c) weight of callus. Bars with asterisk indicate statistical difference at $p \leq 0.05$.*

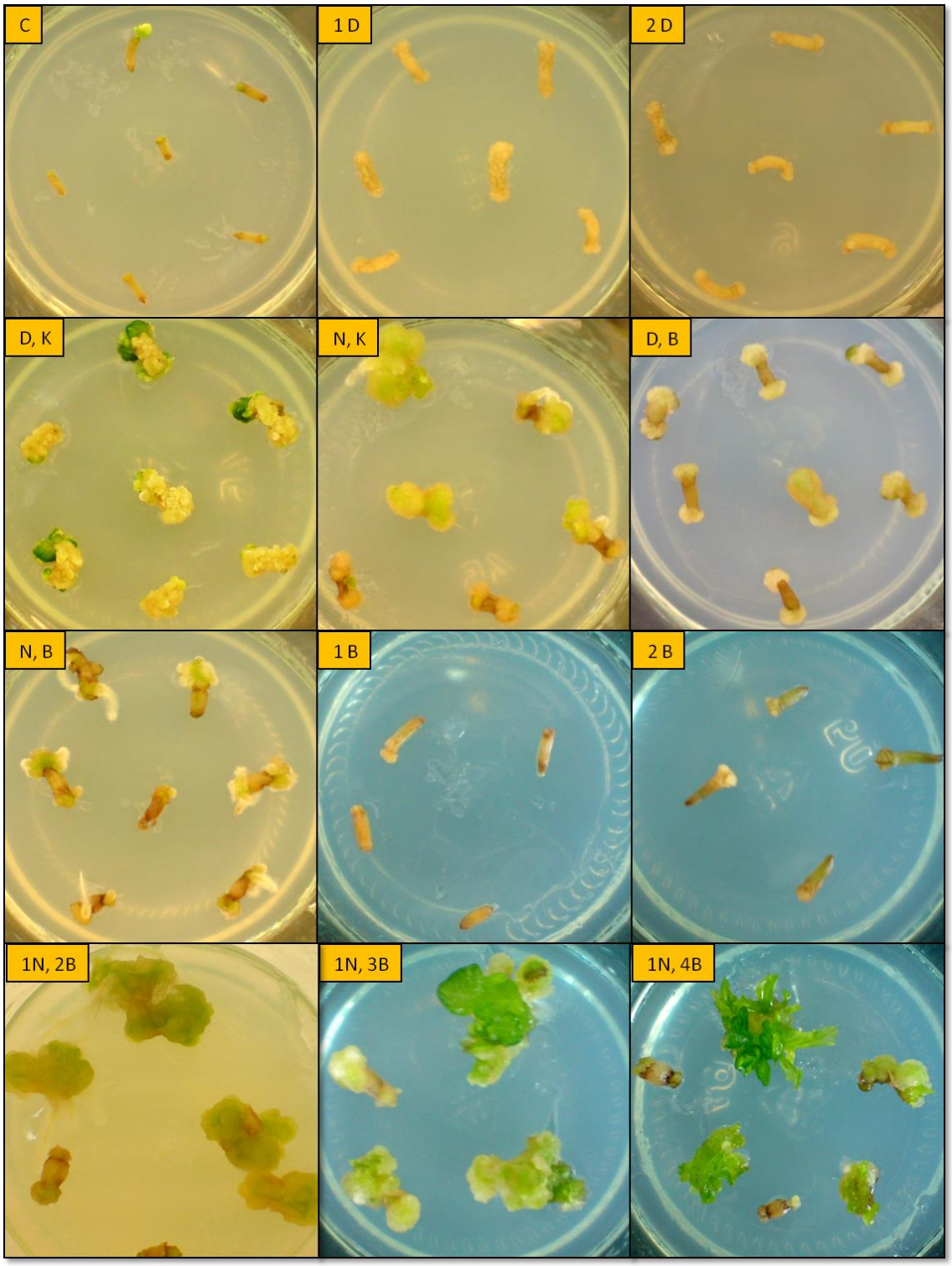

***Figure 2**. Effect of different hormonal combinations on hypocotyl culture of B. napus seedlings after six weeks. This figure is a representative image for Figure 1.*

In some species [29, 32] including some rapeseed cultivars [33, 34] it has been reported that 2, 4-D or its combination with other growth regulators can be the best growth regulator for inducing callus. However, in the present study, FWC and DWC of the calli induced by 2, 4-D or by its combination with other growth regulators were not remarkable, although was significant in some cases compared with control.

Figure 3 shows TEM image from ZnO NPs, verifying the size reported by the manufacturer (<50 nm). As shown, Morphology of ZnO NPs is cube and rod shaped.

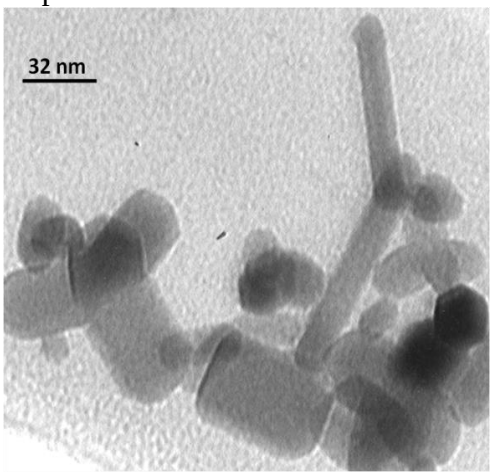

Figure 3. *TEM image of ZnO NPs.*

Results showed that low concentration (10 mg L^{-1}) of ZnO NPs, ZnO BPs or Zn^{2+} did not significantly affect the CP (Figure 4a). However, both FWC and DWC were significantly increased under 10 mg L^{-1} ZnO NPs, while it decreased under the same concentration of ZnO BPs (Figure 4 b, c). Zn^{2+} in the same concentration did not significantly affect the same traits. All other concentrations (25, 50, and 100 mg L^{-1}) of ZnO NPs, ZnO BPs or Zn^{2+} significantly decreased CP, FWC, and DWC in a dose-dependent manner (Figure 4a-c), where under 100 mg L^{-1} ZnO NPs no callugenesis was observed.

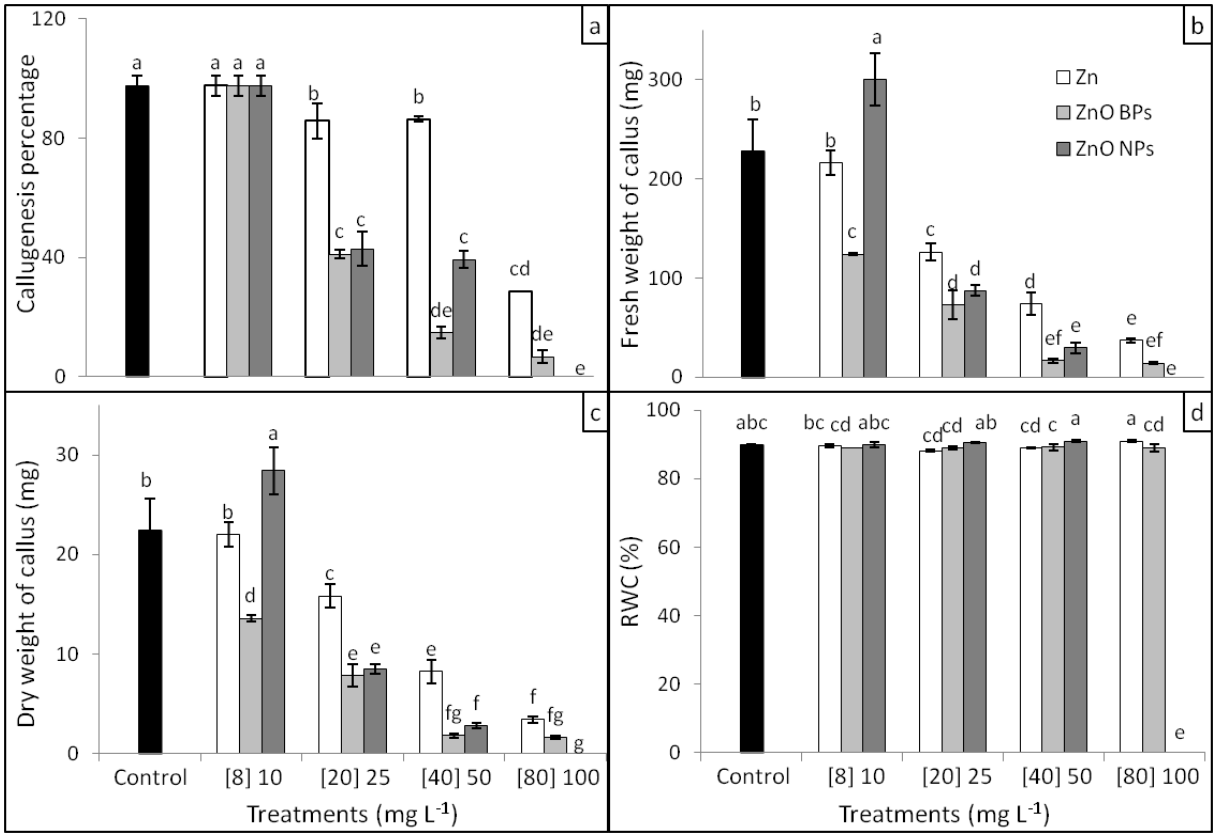

Figure 4. *Effect of ZnO NPs, ZnO BPs, or Zn^{2+} on callugenesis percentage (a), Fresh (b) and Dry (c) weight of callus, and RWC (d). Numbers in the bracket indicate equivalent Zn^{2+} concentrations. Bars with different letters indicate significant difference at $p \leq 0.05$.*

Results also showed that neither of treatments did not significantly affected RWC (Figure 4 d). Surprisingly, while the MS media contained a hormonal combination optimized for callugenesis, those supplemented with 10 mg L^{-1} ZnO NPs

showed a significant SRP. Such regeneration was not induced by other types of treatments (Figure 5).

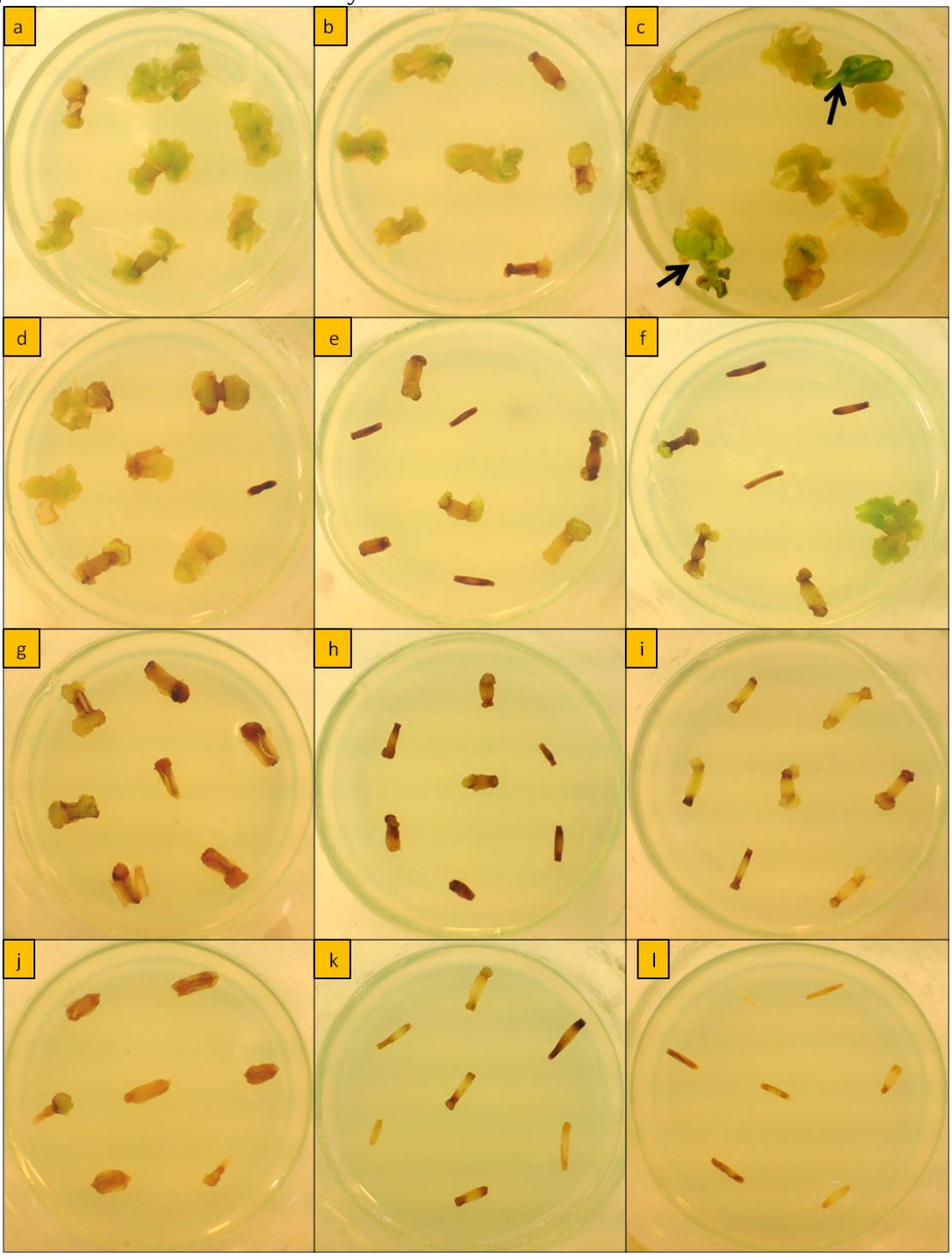

***Figure 5**. Effect of 10 (a-c), 25 (d-f), 50 (g-i), and 100 mg L^{-1} (j-l) of Zn^{2+} (a, d, g, j), ZnO BPs (b, e, h, k), or ZnO NPs (c, f, i, l) on hypocotyl culture of B. napus seedlings after six weeks. Control group is shown in Figure 2. Arrowheads indicate shoot regeneration.*

Few studies have investigated the effect of NPs on plant tissue culture, mainly focused on their benefit in microorganisms decontaminating. For instance, Mandeh et al. [26] studied the effect of TiO_2 NPs on *Hordeum vulgare* tissue culture. They

found that 60 mg L^{-1} TiO_2 NPs could increase callugenesis and the size of calli, and also reduced bacterial and fungal contamination. In another study, Helaly et al. [29] reported the effect of 50, 100, and 200 mg L^{-1} Zn NPs and ZnO particles on biological contamination and organogenic regeneration of banana in vitro cultures. They obtained the contamination-free cultures of banana in MS media supplemented with the same NPs, with no negative effect on regeneration. They also reported that callus growth decreased with increasing the nanoparticles dose. Mahna et al. [27] used Ag NPs as an antimicrobial agent for decontamination of explants, before transferring onto the MS medium. They also reported that preexposure of seeds, leaf, and cotyledon explants to NPs had no effect on the explants viability.

Although investigation of the effects of metal NPs on plant tissue culture is scarce, the effect of metals and their various salts on tissue culture have been widely investigated [35, 30]. For instance, the role of zinc in the growth of plant tissue cultures has been reported many years ago [36]. In a study, the effect of three metal compounds including $ZnSO_4$, $NiSO_4$, and $CuSO_4$ was studied on the growth of secondary callus tissue of *Nicotiana tabacum* L. and *Ruta graveolens* L. and the result showed that the fresh weight of the callus tissue was decreased by the metal compounds in both plant species [37]. Induction of zinc tolerance in the calli of *Setaria italica* L. and regeneration of zinc tolerant calli were investigated by Samantaray et al. [38].

Study on the Effect of different concentrations of copper on tissue culture *of Acer pseudoplatanus* L. showed that callus growth was significantly reduced at 10 mg l^{-1}, with necrosis occurring at 20 mg L^{-1} [39]. In another study, it has been reported that addition of $AgNO_3$ to callus induction medium was significantly effective for shoot regeneration from leaves of rapeseed [31]. Ma et al. [40] were investigated the effects of rare earth elements including lanthanum nitrate (La^{3+}) and cerium nitrate (Ce^{4+}), on callus growth and shoot differentiation of *Echinacea angustifolia*. Their results showed that Low concentrations of La^{3+} and Ce^{4+} (0.01, 0.1 and 1 mgL^{-1}) can increase callus growth while their high concentration (10 and 100 mgL^{-1}) had a suppressive effect on its growth.

In the present study, NPs in low concentration had an inductive effect on the callus growth and subsequently shoot regeneration. Authors speculate that NPs having small size and unique features [41], compared with BPs, can enter the explants, and thus affect some genetic reprogramming features. However, due to the different mechanisms such as generation of ROS and oxidative stress [42], NPs in higher concentration may act as a genotoxic and cytotoxic agent. Kumari et al. [3] investigated cytogenetic and genotoxic effects of 25-100 mgL^{-1} ZnO NPs on root cells of *Allium cepa*, and reported their effects on mitotic index, micronuclei index, chromosomal aberration index, and lipid peroxidation. They concluded that cytogenetic and genotoxic effects of ZnO NPs were due more to their nano size than to ions dissolution or bulk size forms.

4. CONCLUSION

It can be concluded that optimum concentration of ZnO NPs can be beneficial for inducing callus and/or shoot regeneration in the plant tissue culture. Since inductive and inhibitory effects of ZnO NPs were more than those of ZnO BPs and Zn^{2+}, these effects may be due to the NPs entrance into the explants tissues and their effects on different components of cells, including genetic material and program, rather than due to their ion dissolution or their physical interaction on explants surface.

ACKNOWLEDGEMENTS

The authors thank *Mohammad Bagher Erfanian*, PhD student of Ferdowsi

University of Mashhad, for the kindly assistance.

REFERENCES

1. Ahmad, P., Rasool, S., (2014). "*Emerging Technologies and Management of Crop Stress Tolerance*'', Academic Press, USA.
2. Auffan, M., Rose, J., Bottero, J-y., Lowry, G. V., Jolivet, J-P., Wiesner, M. R., (2009). "Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective", *Nat. Nanotechnol.,* 4: 634-641.
3. Kumari, M., Khan, S. S., Pakrashi, S., Mukherjee, A., Chandrasekaran, N., (2011). "Cytogenetic and genotoxic effects of Zn oxide nanoparticles on root cells of *Allium cepa*", *J. Hazard Mater.*, 190: 613–621.
4. Lee, S., Kim, S., Kim, S., Lee, I., (2013b). Assessment of phytotoxicity of ZnO NPs on a medicinal plant, *Fagopyrum esculentum*. *Environ. Sci. Pollut. Res.,* 20: 848–854.
5. Lopez-Moreno, M. L., De La Rosa, G., Hernandez-Viezcas, J. A., Castillo-Michel, H., Botez, C. E., Peralta-Videa, J. R., Gardea-Torresdey, J. L., (2010a). "Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) plants", *Environ. Sci. Technol*., 44: 7315-7320.
6. Lin, D., Xing, B., (2007). "Phytotoxicity of nanoparticles: inhibition of seed germination and root growth", *Environ. Pollut.,* 150: 243–250.
7. Lin, D., Xing, B., (2008). "Root uptake and phytotoxicity of ZnO nanoparticles", *Environ. Sci. Technol.,* 42: 5580–5585.
8. Stampoulis, D., Sinha, S. K., White, J. C., (2009). "Assay-dependent phytotoxicity of nanoparticles to plants", *Environ. Sci. Technol*., 43:9473–9.
9. Lee, C. W., Mahendra. S., Zodrow, K., Li, D., Tsai, Y. C., Braam, J., (2010). "Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana*", *Environ. Toxicol. Chem.,* 29: 669–675.
10. De la Rosa, G., López-Moreno, M. L., Hernández-Viescaz, J., Montes, M. O., Peralta-Videa, J. R., Gardea-Torresdey, J. L., (2011). "Toxicity and biotransformation of ZnO nanoparticles in the desert plants *Prosopis juliflora-velutina*, *Salsola tragus* and *Parkinsonia florida*", *Int. J. Nanotechnol.,* 8 (6/7): 492-506.
11. Ghodake, G. Y., Seo, D., Lee, D. S., (2011). "Hazardous phytotoxic nature of cobalt and Zn oxide nanoparticles assessed using *Allium cepa*", *J. Hazard Mater.,* 186: 952–955.
12. Hernandez-Viezcas, J. A., Castillo-Michel, H., Servin, A. D., Peralta-Videa, J. R., Gardea-Torresdey J. L., (2011). "Spectroscopic verification of Zn absorption and distribution in the desert plant *Prosopis juliflora-velutina* (velvet mesquite) treated with ZnO nanoparticles", *Chem. Eng. J.,* 170: 346–352.
13. Dimkpa, C. O., McLean, J. E., Latta, D. E., Manangon, E., Britt, D. W., Johnson, W. P., Boyanov, M. I., Anderson, A. J., (2012). "CuO and ZnO nanoparticles: phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat", *J. Nanopart. Res*., 14: 1125.
14. Pokhrel, L. R., Dubey, B., (2013). "Evaluation of developmental responses of two crop plants exposed to silver and Zn oxide nanoparticles", *Sci. Total Environ.,* 452–453: 321–332.
15. Lee, S., Chung, H., Kim, S., Lee, I., (2013a). "The genotoxic effect of ZnO and CuO nanoparticles on early growth of buckwheat, *Fagopyrum Esculentum*", *Water Air Soil pollut.,* 224:1668.
16. Rao, S., Shekhawat, G. S., (2014). "Toxicity of ZnO engineered nanoparticles and evaluation of their effect on growth, metabolism and tissue specific accumulation in *Brassica juncea*", *J, Environ. Chem. Eng.,* 2: 105–114.
17. Yoon, S. J., Kwak, J. I., Lee, W. M., Holden. P. A., An, Y. J., (2014). "Zinc oxide nanoparticles delay soybean development: A standard soil microcosm study", *Ecotoxicol. Environ. Saf.,* 100: 131–137.
18. Mousavi Kouhi, S. M., Lahouti, M., Ganjeali, A., Entezari, M. H., (2014). "Comparative phytotoxicity of ZnO nanoparticles, ZnO microparticles, and Zn^{2+} on rapeseed (*Brassica napus* L.): investigating a wide range of concentrations", *Toxicol. Environ. Chem.,* 96 (6): 861-868.
19. Mousavi Kouhi, S. M., Lahouti, M., Ganjeali, A., Entezari, M. H., (2015a). "Comparative effects of ZnO nanoparticles, ZnO bulk particles, and Zn^{2+} on *Brassica napus* after long-term exposure: Changes in growth, biochemical compounds, antioxidant enzyme activities, and Zn bioaccumulation", *Water Air Soil Pollut.,* 226: 364-373.
20. Mousavi Kouhi, S. M., Lahouti, M., Ganjeali, A., Entezari, M. H., (2015b). "Long-term exposure of rapeseed (*Brassica napus* L.) to ZnO nanoparticles: anatomical and ultrastructural responses", *Environ. Sci. Pollut. Res.,* 22 (14): 10733-10743.
21. Nair, R., Varghese, S. H, Nair, B. G., Maekawa, T., Yoshida, Y., Kumar, D. S., (2010). "Nanoparticulate material delivery to plants", *Plant Sci.,* 179: 154–163.
22. Mude, N., Ingle, A., Gade, A., Rai, M., (2009). "Synthesis of Silver Nanoparticles Using Callus Extract of *Carica papaya* - A First Report", *J. Plant Biochem. Biotechnol.,* 18(1): 83–86.

23. Nabikhan, A., Kandasamy, K., Raj, A., Alikunhi, N. M., (2010). "Synthesis of antimicrobial silver nanoparticles by callus and leaf extracts from saltmarsh plant, *Sesuvium portulacastrum* L.", *Colloids Surf. B Biointerfaces.*, 79(2):488-93.
24. Malabadi, R. B., Chalannavar, R. K., Meti, N. T., Mulgund, G. S., Nataraja, K., Vijaya Kumar, S., (2012). "Synthesis of antimicrobial silver nanoparticles by callus cultures and in vitro derived plants of *Catharanthus roseus*", *Res. Pharm.*, 2(6): 18-31.
25. Hegazy, H. S., Rabie, G. H., Shaaban, L. D., Raie, D. S., (2014). "Extracellular Synthesis of Silver Nanoparticles by Callus of *Medicago sativa*", *Life Sci. J.* 11(10): 1211-1214.
26. Mandeh, M., Omidi, M., Rahaie, M., (2012). "In Vitro Influences of TiO2 Nanoparticles on Barley (*Hordeum vulgare* L.) Tissue Culture", *Biol. Trace Elem. Res.*, 150:376–380.
27. Mahna, N., Vahed, S. Z., Khani, S., (2013). "Plant In vitro Culture goes Nano: Nanosilver-Mediated Decontamination of Ex vitro Explants", *J. Nanomed. Nanotechol.*, 4: 161.
28. Arab, M. M., Yadollahi, A., Hosseini-Mazinani, M., Bagheri, S., (2014). "Effects of antimicrobial activity of silver nanoparticles on in vitro establishment of G · N15 (hybrid of almond · peach) rootstock", *J. Genet. Eng. Biotechnol.*, 12: 103–110.
29. Helaly, M. N., El-Metwally, M. A., El-Hoseiny, H., Omar, S. A., El-Sheery, N. I., (2014). "Effect of nanoparticles on biological contamination of in vitro cultures and organogenic regeneration of banana", *AJCS.*, 8 (4): 612-624.
30. Murashige, T., Skoog, F., (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures", *Physiol, Plant.*, 15: 473–497.
31. Akasaka-Kennedy., Y., Yoshida, H., Takahata, Y., (2005). "Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of $AgNO_3$ and genotype". *Plant Cell Rep*., 24: 649–654.
32. Murata, M., Ortan. T. J., (1987). "Callus initiation and regeneration capacities in Brassica species", *Plant Cell Tissue Organ Culture.*, 11:111-123.
33. Jonoubi, P., Mousavi, A., Majd, A., Salmanian, A. H., Jalali Javaran, M., Daneshian, J., (2005). "Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*", *Biol. Plant.*, 49 (2): 175-180.
34. Maheshwari, P., Selvaraj, G., Kovalchuk, I., (2011). "Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation", *New Biotechnol.*, 29 (1):144-55.
35. Klein, R. M., Manos, G. E., (1960). "Use of metal chelates for plant tissue cultures", *Ann. New York Acad Sci.*, 88: 416–425.
36. Klein, R. M., Caputo, E. M., Witterholt, B. A., (1962). "The Role of Zinc in the Growth of Plant Tissue Cultures", *Am. J. Bot.*, 49 (4) 323-327.
37. Maróti, M., Bognár, J., (1988). "Effect of heavy metals on the growth of tissue cultures (II)", *Acta Biol. Hung.*, 39(1): 75-85.
38. Samantaray, S., Rout, G. R., Das, P., (1999). "In vitro selection and regeneration of zinc tolerant calli from *Setaria italica* L.", *Plant Sci.*, 143: 201–209.
39. Turner, A. P., Dickinson, N. M., (1993). Copper tolerance of Acer pseudoplatanus L. (sycamore) in tissue culture", *New Phytol.*, 123 (3): 523–530.
40. Ma, L., Zhao, J., Wu, H., Yuan, X., Hou, D., (2010). "Effects of rare earth elements on callus growth, soluble protein content, peroxidase activity and shoot differentiation of Echinacea angustifolia cultures in vitro", *Afr. J. Biotechnol.*, 9(16) 2333-2341.
41. Ayatollahzade Shirazi, M. S., Ramezanpour, Z., (2016). "Toxicity effects of SiO_2 nanoparticles on green micro-algae *Dunaliella Salina*", *Int. J. Nanosci. Nanotechnol.*, 12(4) 269-275.
42. Zarafshar, M., Akbarinia, M., Askari, H., Hosseini, S. M., Rahaie M., Struve, D., (2015). "Toxicity assessment of SiO_2 nanoparticles to pear seedlings", *Int. J. Nanosci. Nanotechnol.*, 11(1) 13-22.