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Research Article

In vitro screening of Nano ZnO particles on date palm (*Phoenix dactylifera* L.)

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Abstract | Current study aimed to check the influence of nano ZnO particles on removal of microbial contaminants for *in vitro* protocol establishment of *Phoenix dactylifera* L. The contamination-free date palm plantlets were achieved by the addition of ZnO nanoparticles at various concentrations (0.04, 0.06 & 0.08 mg/l) into MS basal medium supplemented with cytokinin (BAP1). Immature apical meristems of *P. dactylifera* L. were used as explant. While using different concentrations of nanoparticles it was suggested that minimum shoot induction time and maximum shoot length showed with 0.06 mg/l. Results demonstrated a positive effect on regeneration. The best media for rooting was determine to be BAP1+NAA0.5 mg/l. Redeveloped plantlets were positively hardened and showed about 95% productivity in compost+sand+biofert mixture (1:1:1) when treated with Hoagland nutrient solution on weekly basis. To examine the genetic strength of dates *in vitro* plantlets, further screening of nanoparticles is suggested.

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1. Introduction

Bacterial and fungal agents are the main culprits behind contaminations in *in vitro* plant proliferation and plant tissue culture processes such as callus formation, inoculation of ex-plant and their subculturing. The contamination rates increase during subculturing due to either inadequate or improper sterilization of the explant, media, laboratory equipment and apparatus (Omamor *et al.*, 2007). In date palm micropropagation large amount of explants are demolished because of endogenous bacterial and fungal contaminations. Additionally, the media

used for culturing plant tissue is a rich mixture of nutrients which itself is the major source of microbial contamination. Some pathogens produce poisonous substances or toxins which reduce shoot proliferation and rooting (Helaly *et al*., 2014; Javed *et al*., 2017). In general, surface sterilization of the explants removes surface contaminants only instead of the endogenous microbes therefore contaminations on the base or around explants are a big hurdle in micropropagation. Removal of such contamination is facilitated by antibiotics and antifungal agents in the growth media of plant cultures (Habiba *et al*., 2002).

Plant nano-biotechnology is developing with tremendous prospective towards plant enhancement. The addition of nanoparticles in growth medium of plant tissue culture affects callus formation, shoot multiplication, and root induction by modifying antioxidant activities such as enzyme actions, gene expression, removal of production of ethylene (Kim *et al*., 2017). The Nano ZnO particles are anti-microbial agents that conquers their growth. They are generally active against fungal as well as both gram-positive and gram-negative bacteria contaminants (Helaly *et al*., 2014).

Date palm is one of the oldest cultivated trees which has been used as a dietary supplement for many centuries around the world (Alfaro-Viquez *et al*., 2018). The date fruit has a high nutritional value, it contains large amount of inorganic salts, vitamins and sugars which comprise almost 70% of the fruit, therefore making it an excellent source of energy (Yen *et al*., 2018). An estimated worldwide production of date palm is more than 3,000 cultivars (Moussouni *et al*., 2017). Pakistan as the 5th largest global importer of dates, imports 19,777 tons annually whereas the total indigenous date production is 650,000 metric tons. Pakistan is the 2nd largest exporter of *Phoenix dactylifera* L. with 104,090 tons per annum (Khan and ul Haq, 2022). The provincial production of dates in Sindh is 45.4%, Balochistan is 44.8%, Punjab is 7.9% and Khyber Pakhtunkhwa is 1.9% (Baloch *et al*., 2014). Almost 85% of dates production in Pakistan come from district of Khairpur of Sindh (Abul-Soad, 2010). Sindh is dominated by Asul khurmo, Aseel, Autaqin, Karbalian, Khar, Fasli, Bhedir, Mithri, Dedhi, Began, Kupro, Gajjar, and Kachoo wari. While, Punjab cultivations are Khudravi, Zahidi, Hilawi and Shamran. On the other hand, in Baluchistan there are Muzawati, Begum Jangi, Rabai, Shakri, Sabzo, Aab-e-dandan, Hussaini, Kehraba, Jaan Swore and Halini (Zaid and De Wet, 1999).

Many date plantations are destroyed by disease “sudden decline syndrome” at Sind region. The infection rate is increased day by day. This disease somewhat resembles the Palm Lethal Yellowing disease produced by *Phytoplasma* and Bayoud disease produced by *Fusarium oxysporum* reported in Algeria and Morocco (Abul-Soad *et al*., 2011). To overcome this disease, plant tissue culture technique is used to produce a large number of palm clones (Al Kaabi *et al*., 2001). Application of tissue culturing for *P. dactylifera* L. is termed as *in vitro* propagation (Zaid and De Wet, 1999). Therefore, the purpose of current work was to study the synergistic effect of nanotechnology and micropropagation techniques by using ZnO nanoparticles and different plant growth regulators to eradicate microbial contaminations for the production of dates plantlets.

2. Materials and Methods

2.1. Collection of plant seeds

Dates seeds (Medjool) were taken from Metro store, Lahore. All seeds were from fertilized and mature fruits.

2.2. Sterilization of seeds

Date seeds were soaked in distilled water overnight (12 hours). Seeds were washed thoroughly to remove any adhered materials. After rinsing, a few drops of liquid soap were added then shaken vigorously. Seeds were again washed with double distilled water to eliminate any traces of bleach or detergent. Seeds were immersed in concentrated sodium hypochlorite for 1 hour. Seeds were finally washed after an hour, then autoclaved.

2.3. Preparation of explant

Date seeds were inoculated in MS basal medium (Murasnige and Skoog, 1962) containing 50ml macronutrients, 10ml micronutrients, 10ml vitamins, 5ml Fe-EDTA and 30g/l sugar. MS medium was fortified with various concentrations of cytokinin and auxin. Before autoclaving pH was adjusted to 5.5-5.7. These cultured tubes were placed in culture chamber with specific conditions to incubate them at 20±2°C with 16-hour photoperiod having 2000-3000 lux light intensity whereas the dark period provided was precisely 8 hours. Germination days and shoot initiations were also observed

2.4. Inoculation of Immature apical meristem

The immature apical meristems which arose from the base of germinated seeds (5mm) were excised and inoculated into MS medium supplemented by different growth promoters (cytokinin, combination of cytokinins and auxins). After selecting medium for the best growth, various amounts of nano ZnO particles (0.04, 0.06 and 0.08 mg/l) were added to the media to evaluate the antibacterial and antifungal potential of nanoparticles on *in vitro* grown plantlets.

2.5. Acclimatization of plantlets

After rooting, the plantlets were shifted to small pots containing different proportions of compost+sand+biofert in the growth chamber (El Kinany *et al.*, 2018). The plantlets were treated with Hoagland solution weekly. After one month the acclimatized plantlets were transferred into green-house under controlled conditions for further multiplications.

2.6. Statistical Analysis

For the current experiment a completely randomized design was applied. The statistics were examined by "Analysis of Variance" (ANOVA). Data was analysed after five experiments, all were replicated based on "Duncan's New Multiple Range Test", that the null hypothesis was ≤0.05% using the "SPSS" version 20.0.

3. Results

Various concentrations of BAP showed varying effects on shoot induction in explant of *P. dactylifera* L. (Table 1). The best result of shoot initiation (96%) was examined in BAP1 mg/l in which shoot initiated within 5±0.39e days after inoculation and gained a maximum size of 18.2±0.09a cm. Keeping the concentration of Kinetin 0.5 mg/l constant with gradual increase of BAP ranged 1-5 mg/l, a variation in the days for shoot multiplication was seen. Table 2 shows the maximum result of shoot initiation (90%) examined in BAP1 + Kin0.5 mg/l in which shoot initiated within 5±0.38c days and the shoot gained a maximum height of 12±0.36a cm along with 2±0.18b number of shoots.

Table 1: Role of BAP (mg/l) on shoot regeneration

Medium concentration BAP (mg/l)	Time for shoot induction (days)	Frequency of shoot induction (%)	Shoot length (cm)	Number of shoots
0.0	9±0.52^{a}	71	9.5±0.18^{e}	1±0.19^{b}
0.5	6±0.23^{d}	73	11.7±0.29^{d}	1±0.09^{b}
1.0	5±0.39^{e}	96	18.2±0.09^{a}	3±0.06^{a}
2.0	7±0.19^{c}	85	14.4±0.40^{c}	2±0.33^{b}
3.0	8±0.46^{b}	86	15.7±0.25^{b}	1±0.36^{a}

Table 2: Role of BAP+Kinetin mg/l on plantlets regeneration

Plant Growth Regulators		Time for shoot induction (Days)	Frequency of shoot induction (%)	Shoot length (cm)	No. of shoots
BAP (mg/l)	Kinetin (mg/l)				
0.5	0.5	6±0.23^{b}	82	11.7±0.18^{a}	1±0.20^{a}
1	0.5	5±0.38^{c}	90	12±0.36^{a}	2±0.18^{b}
2	0.5	6±0.10^{b}	78	11±0.23^{b}	1±0.10^{b}
3	0.5	8±0.22^{a}	76	9±0.17^{c}	1±0.30^{b}

Different parameters were utilized in combinations of various amounts of BAP (ranged 1- 5 mg/l) and fixed concentration of IAA (0.5 mg/l) to observe their effects on shoot length. Table 3 depicts that the highest length of shoot was recorded at 10.1±0.05a cm within 6±0.12c days. It also showed 90% frequency for shoot length induction was achieved in media concentrations of BAP1 + IAA0.5 mg/l.

Table 3: Role of BAP+IAA mg/l on shoot initiation

Plant Growth Regulators		Time for shoot induction (Days)	Frequency of shoot induction (%)	Shoot length (cm)	Numbers of shoots
BAP (mg/l)	IAA (mg/l)				
0.5	0.5	7±0.11^{b}	81	9.2±0.08^{b}	1±0.11^{b}
1	0.5	6±0.12^{c}	90	10.1±0.05^{a}	2±0.11^{a}
2	0.5	7±0.10^{b}	78	8.7±0.05^{c}	1±0.10^{b}
3	0.5	8±0.13^{a}	76	6.2±0.07^{d}	1±0.05^{b}

The immature apical meristems were inoculated into several volume of BAP (mg/l) in fixed concentrations 0.5mg/l of IBA for shoot regeneration studies. Table 4 shows that the highest regenerated shoot length acquired was 10.1±0.07a cm having number of shoots within 6±0.14bc days. It also showed 90% frequency for shoot regeneration at media BAP1+IBA0.5 mg/l.

Table 4: Role of BAP+IBA mg/l for shoot regeneration

Plant Growth Regulators		Time for shoot induction (Days)	Frequency of shoot induction (%)	Shoot length (cm)	Number of shoots
BAP (mg/l)	IBA (mg/l)				
0.5	0.5	9±0.15^{a}	72	6.3±0.12^{c}	1±0.11^{b}
1	0.5	6±0.14^{bc}	90	10.1±0.07^{a}	2±0.31^{a}
2	0.5	6±0.2^{c}	78	7.2±0.21^{b}	1±0.33^{b}
3	0.5	7±0.5^{b}	81	7.5±0.22^{b}	1±0.23^{b}

In Table 5 the outcome of different volume of BAP ranged 1-5 mg/l and fixed concentrations 0.5 mg/l of NAA used to observe growth of *P. dactylifera* L. plantlets. The superlative result was 94% in BAP1+NAA0.5 mg/l in which shoot developed after 5±0.09c days and gained a maximum length of 14.3±0.13a cm along with 2±0.19a number of shoots. It was observed that the combination of BAP + NAA induced rooting in dates palm *in vitro* plantlets in which 3±0.11a roots generated with 3.4±0.12a cm in size.

Table 5: Role of BAP+NAA mg/l for shoot regeneration

Plant Growth Regulators		Time for shoot induction (Days)	Frequency of shoot induction (%)	Shoot length (cm)	No. of shoots	Roots length (cm)	Number of roots
BAP (mg/l)	NAA (mg/l)						
0.5	0.5	9±0.11^{a}	74	10.1±0.16^{b}	1±0.15^{b}	0.7±0.05^{d}	1±0.14^{c}
1	0.5	5±0.09^{c}	94	14.3±0.13^{a}	2±0.19^{a}	3.4±0.12^{a}	3±0.11^{a}
2	0.5	7±0.38^{b}	84	8.8±0.17^{c}	1±0.20^{b}	2.6±0.17^{b}	2±0.26^{b}
3	0.5	7±0.20^{b}	80	7.2±0.12^{d}	1±0.10^{b}	1.4±0.10^{c}	1±0.29^{c}

Various growth parameters such as shoot regeneration time, percentage frequency and length of shoots were examined when the BAP concentration was gradually changed from 1-5 mg/l with fixed amount 0.5 mg/l of Kin + IAA (Figure 1 and 2). A variation in the days for shoot induction and the shoots length was observed in which shoot initiated within 7±0.12d days of inoculation and gained a maximum length of 16.2±0.10a cm in 5 months along with 3±0.10a number of shoots in BAP 1.0 + Kin 0.5 + IAA 0.5 mg/l.

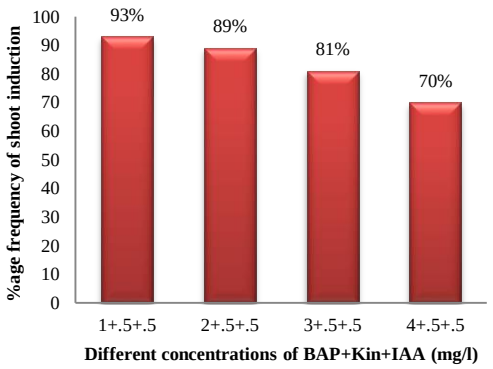

Figure 1: Effect of combination of different cytokinins + auxin on percentage frequency of shoots induction.

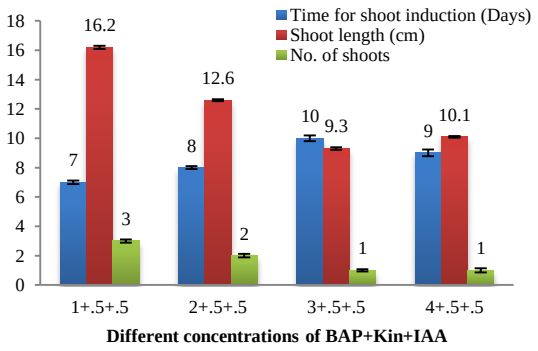

Figure 2: Effect of combination of different cytokinins + auxin on

percentage frequency of shoot length, time and numbers.

Combinations of different cytokinin and auxin showed different results in shoot induction and shoot multiplications. The combination of various quantities of BAP and constant concentration 0.5 mg/l of Kin+NAA was analyzed for shoot regeneration (Figure 3, 4 and 5). The greatest outcomes were obtained in BAP1 + Kin0.5 + NAA0.5 mg/l as the shoot development was 5±0.10c days of inoculation and the shoot gained a maximum length of 14.7±0.06a cm along with 3±0.06a number of shoots. It is also observed that in this 2±0.05a roots generated with 3.6±0.06a cm in length.

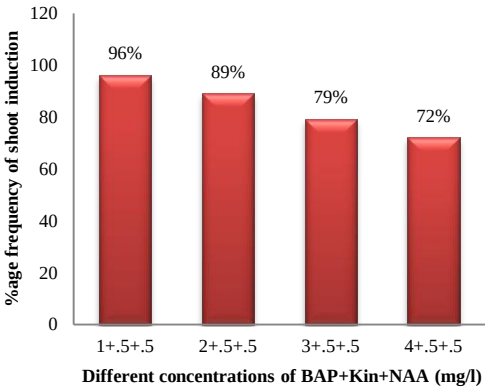

Figure 3: Effect of different concentrations of cytokinins + auxin on percentage frequency of shoot induction in date germination

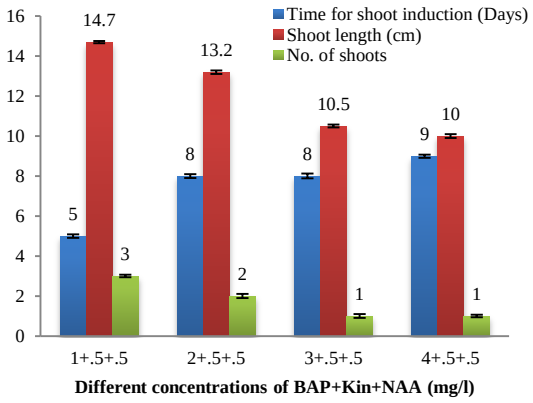

Figure 4: Effect of different concentrations of cytokinins + auxin on percentage frequency of time, length and number of shoots in date germination

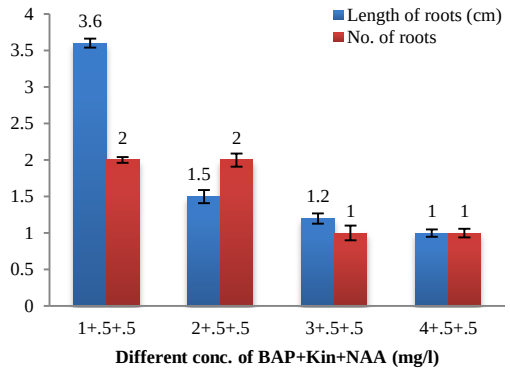

Figure 5: Effect of different concentrations of cytokinins + auxin on percentage frequency of root initiation in date germination

In the present study the constant concentration of BAP1 mg/l and different concentrations of nanoparticles ranged 0.04, 0.06 and 0.08mg/l were used. The best result of shoot germination was 96% in BAP1.0+0.06 mg/l nanoparticles as shown in Figure 6, 7 and 8. The time of shoot initiation within 4±0.11c days of inoculation and the shoot gained a maximum length of 19.2±0.06a cm. The control medium which was not supplemented with nanoparticles did not produce roots. The average root length was 3.6±0.06a cm and 3±0.06a roots were germinated on nano ZnO particles containing medium.

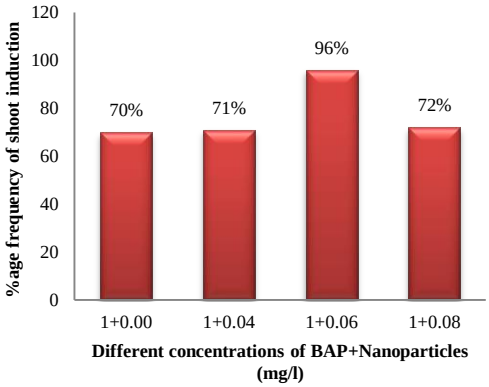

Figure 6: Effects of different concentrations of nanoparticles + BAP on explant percentage frequency of shoot induction

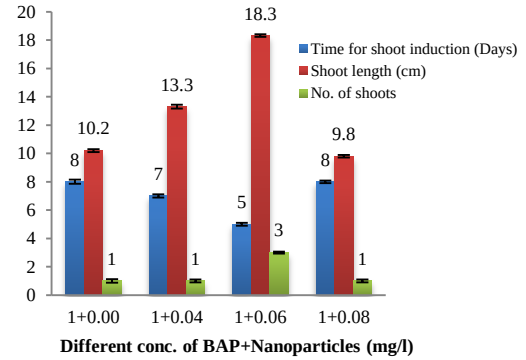

Figure 7: Effects of different concentrations of nanoparticles + BAP on explant time, length and number of shoots

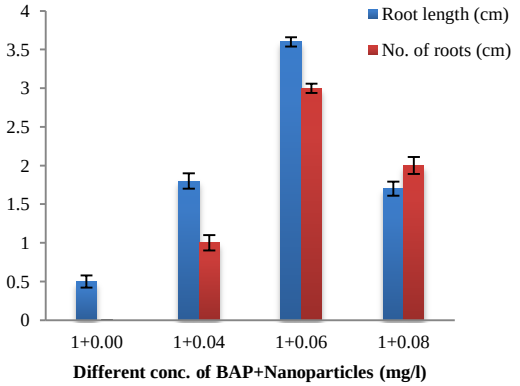

Figure 8: Effects of different concentrations of nanoparticles + BAP on explant length and number of roots

In vitro micropropagated plantlets of *P. dactylifera* L. were shifted to greenhouse in different kinds of potting mixture which were sand + cocopeat, leaf manure + cocopeat, sand + soil + leaf manure, and compost + sand + biofert as shown in Table 6. The plants survival frequency was best (95%) in compost+sand+biofert when treated with Hoagland solution on regular basis.

Table 6: Effect of different concentrations of ptting media on acclimatization of *in vitro* regenerated plantlets of *P. dactylifera* L.

Potting media	Mixing Ratio	Frequency of plant survival (%)	Growth of plants after acclimatization	
			Growth of plants treated with Hoagland solution	Growth of plants treated without Hoagland solution
Sand + soil + leaf manure	1:1:1	70	+	+
Sand + cocopeat	1:1	80	++	+
Cocopeat + leaf manure	1:1	85	++	+
Cocopeat + sand + biofert	1:1:1	95	+++	++

Excellent +++ Good ++ Fair +

4. Discussion

Current work was designed to study the synergistic effect of nanotechnology and micropropagation techniques by using ZnO nanoparticles and different plant growth regulators to eradicate microbial contaminations for the production of date plantlets. In current work maximum result of date shoot initiation (90%) was examined in BAP1 + Kin0.5 mg/l in which shoot initiated within 5±0.38c days and the shoot gained a maximum height of 12±0.36a cm along with 2±0.18b number of shoots. These results are almost identical to those presented by Hegazy and Aboshama (2010). Furthermore, in present work the highest recorded shoot length was 10.1±0.05a cm attained within 6±0.12c days. This was achieved in media concentrations of BAP1 + IAA0.5 mg/l. These observations are in accordance with those reported by Qaddoury and Amssa (2004).

Shoot regeneration studies were performed on immature apical meristems inoculated into several volume of BAP (mg/l) in fixed concentrations 0.5mg/l of IBA. The highest shoot length attained was 10.1±0.07a cm within 6±0.14bc days in BAP1+IBA0.5 mg/l media. Similar procedure and results were yielded in

works reported by Mazri (2012) and Bekheet (2013).

The growth of *P. dactylifera* L. plantlets was observed at 94% efficiency in BAP1+NAA0.5 mg/l where the shoot developed after 5±0.09c days and gained a maximum length of 14.3±0.13a cm along with 2±0.19a number of shoots. The combination of BAP + NAA induced rooting in dates palm in *in vitro* plantlets in which 3±0.11a roots generated with 3.4±0.12a cm in size. The role of NAA in root formation has been previously recognized by other researchers (Khierallah and Bader, 2006; Khan and Bi, 2012; Jazinizadeh *et al*., 2015; Wang *et al*., 2022). Therefore, for rooting the best auxin was NAA rather than IBA or IAA.

Growth parameters such as shoot regeneration time, percentage frequency and length of shoots were examined in present study. The shoot initiated within 7±0.12d days of inoculation and gained a maximum length of 16.2±0.10a cm in 5 months along with 3±0.10a number of shoots in BAP 1.0 + Kin 0.5 + IAA 0.5 mg/l. Similar observation about shoot development were reported by (Meziani *et al*., 2015).

In current work the maximum shoot length was obtained in BAP1 + Kin0.5 + NAA0.5 mg/l as the shoot development was 5±0.10c days of inoculation and the shoot gained a maximum length of 14.7±0.06a cm along with 3±0.06a number of shoots. It is also observed that in this 2±0.05a roots generated with 3.6±0.06a cm in length. Similar facts were proposed by Khan and Bi (2012) and Bekheet (2013).

In present study, the regeneration potential increased with nano ZnO which may be due to the effect of Zn on plant growth. The Zn is an essential element for plants however it is toxic at high levels (Paschke *et al*., 2006). It was deducted from the outcomes of present work that medium fortified with BAP, Kin and NAA were the best combinations for shoot development, these results are in accordance with Helaly *et al*. (2014).

The *P. dactylifera* L. plantlets were shifted to greenhouse, it was observed that the best plant survival frequency (95%) was achieved in potting mixture composed of compost+sand+biofert, when regularly treated with Hoagland solution. The acclimatization of *in vitro* grown plantlets was also studied by El Kinany *et al*. (2018) and corroborated in agreement with present work. However, some researchers used cow manure instead of biofert with similar outcomes, as reported by Kurup *et al*. (2014).

5. Conclusions

It was determined that BAP1 mg/l was the best media for date growth. Nanoparticle concentration of 0.06 mg/l fortified with BAP1 produced the most encouraging results in increasing the regeneration rate of explant. Additionally it had a noteworthy control on microbial contaminants and enhanced plant growth. Low concentration of nanoparticles showed no effect on regeneration of plantlets however, high concentration of nanoparticles showed negative effect of *in vitro* date plantlets. To examine the genetic strength of date *in vitro* plantlets, further screening of nanoparticles is required.

6. Acknowledgments

Authors would like to acknowledge Plant Tissue Culture Laboratory, Bagh-e-Jinnah, Lahore for providing lab and facilities in conducting present work.

7. Author's Contribution

All authors have made a substantial contribution to the concept, design,

analysis and interpretation of data for the article.

8. Conflict of Interest

There was no conflict of interest among authors regarding the publication of this article

9. Novelty Statement

Present study explored the synergistic effects of nanotechnology and micropropagation techniques by using ZnO nanoparticles and different plant growth regulators to eradicate microbial contaminations for the production of date plantlets.

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