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Enhancing Callus Initiation and Salinity Tolerance in Egyptian GZ650 Maize (*Zea mays* L.) Using Zinc Oxide Nanoparticles

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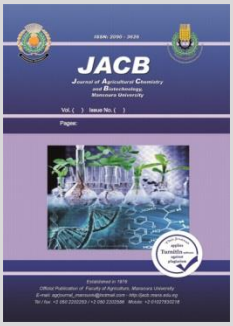

ABSTRACT

This study explored callus initiation and somatic embryogenesis within GZ650 maize genotype, emphasizing the influence of distinct culture media and zinc oxide nanoparticles (ZnONPs). In this experiment, 800 immature embryos were cultured on four different media (control media and different concentrations of zinc media) (CM, ZM1, ZM2, and ZM3). Successful somatic embryogenesis was achieved, with ZM3 medium containing 4000 ppm ZnONPs demonstrating optimal callus induction. ZnONPs consistently outperformed the control in promoting embryogenic callus formation, notably with ZM3 resulting in 89.5% embryogenic calli compared to the control's (43%). Salinity treatments affect ZM3-derived embryogenic calli as well as callus performance. Genetic diversity analysis using inter-simple sequence repeats (ISSR) markers highlighted substantial variations and relationships among maize samples under salinity treatments. This research underscores the potential of ZnONPs to facilitate callus development, enhance plant tolerance against salinity, and assess genetic variation in response to ZnONPs and salinity stress.

Keywords: embryogenic callus, plant regeneration, Zinc oxide nanoparticles, salinity stress, ISSR.

INTRODUCTION

Maize (*Zea mays* L.), a major cereal crop, holds significant value in countries where farming is vital. It's the third most produced cereal after wheat and rice, grown widely across different types of farming areas (FAO 2019). Maize is important because it provides food, feed for animals, oil, and materials for biofuel. However, among the various challenges nature poses, saltiness in the soil is a big problem, especially in dry areas where farming is tough. This gets worse as there isn't much water, more food is needed worldwide, and cities keep growing, pushing farming into less suitable lands (Farnia and Omidi 2015).

Even though we need to grow more maize to feed the growing global population, we lose a lot of it every year because of the saltiness in the soil. This is expected to get worse with changes in the climate (Elsayed *et al*., 2023, Ferguson 2019, Webber *et al*., 2018). As the weather becomes hotter and there's less rain, it becomes harder to balance the needs of people and the needs of crops for water (Lobell *et al*., 2014).

Improving crop plants' ability to handle salinity stress is crucial to enhance productivity when water is scarce and salt levels are high. Recently, focus has shifted towards employing harmless nanoparticles to genetically enhance certain crops, leading to a significant surge in nanoparticle usage. In agriculture, nanoparticles such as (Se), (Au), (Ag), (Si), and (Mg) are employed as fertilizers. Additionally, nanoparticles like copper oxide (CuO), (Fe3O4), (S), chitosan, and calcium-alginate-chitosan nanoparticles function as pesticides or vehicles for delivering pesticides (Ale *et al*., 2021; Bano *et al*., 2021; Singh *et al*., 2021).

In addition to various other nanoparticles, (CuO), (Ag), (Zn), and Fe nanoparticles have demonstrated the capacity to enhance plant growth. However, it's important to note that they have also been associated with phytotoxic effects (Akbarnejad-Samani *et al*., 2020; Fedorenko *et al*., 2021; Goswami *et al*., 2019). The way these nanoparticles work varies across different environments due to differences in physical and chemical conditions (Javed *et al*., 2019; Ghafari and Razmjoo, 2013; Levard *et al*., 2012).

We've seen that using nanoparticles can help grow different plants better, make seeds grow faster, help with changing plant genes, make more useful plant stuff, and protect plants (Hegazi *et al*., 2018; Alsuwayyid *et al*., 2022).

Zinc oxide nanoparticles (ZnONPs) are important for plants and animals. For plants, they help with the chemical processes, like making enzymes work and helping with protein, fat, sugar, and DNA work. These nanoparticles are like helpers for about 200 enzymes and other molecules that do plant work. They also help with building proteins and sugars in plants (Marschner, 1986). ZnONPs also help plants grow better, protect cells from bad chemicals, and save important parts of cells from breaking (Kabata-Pendias, 1999; Cakmak, 2000).

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Out of the many techniques used to study molecules in plants, one that stands out is PCR-based markers, specifically the inter-simple sequence repeat (ISSR) method. This approach has gained wide recognition and success due to its speed, affordability, and the fact that it doesn't require advanced knowledge of DNA sequences. Also, it requires only small amounts of DNA for analysis (Dar *et al*., 2018; Soliman *et al*., 2021).

The present study has been carried out to achieve four main objectives: investigate the effects of zinc oxide nanoparticles (ZnONPs) on callus induction and regeneration of Gz650 Egyptian maize inbred line immature embryos; examine the effect of different sodium chloride (NaCl) concentrations as salinity stress selection on maize calli; analyze the impact of salinity stress on the chemical composition of regenerated maize plantlets; and assess the genotoxic effect and genetic variation in response to ZnONPs and salinity stress.

MATERIALS AND METHODS

Plant material:

The Gz650 Egyptian maize (*Zea mays* L.) inbred line used in this study was obtained from the Maize Department, Field Crops Research Institute, ARC, Giza, Egypt

Callus Induction:

For callus induction, ears were collected from field-grown inbred plants around 10-15 days after self-pollination. The ears were sterilized for 20-minute using a 50% chlorox solution containing 0.1% Tween 20, followed by multiple rinses with autoclaved distilled water under sterile conditions. A total of 800 immature embryos, ranging from 0.5 to 1.0 mm in length, were aseptically extracted from the kernels and placed on callus induction media.

Callus Culture and Nanoparticle Treatment:

To initiate and maintain callus cultures, four different vitamin-based media, (Control media and different concentration Zinc media) CM, ZM1, ZM2, and ZM3, were employed according to Miranda-Fuentes *et al*., (2021). These media contained 2.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 100 mg/l casein hydrolysate, and varied concentrations of zinc oxide nanoparticles (ZnONPs) (1000, 2000, and 4000 ppm) in ZM1, ZM2, and ZM3, respectively. The sucrose concentration in all media was 3%, with a pH of 5.8. Cultures were maintained in darkness at 28°C under a 16/8-hour photoperiod from cool-white, fluorescent lights at 2000 lux. Embryogenic tissues were sub-cultured every 14 days.

Nanoparticles synthesizing:

ZnONPs were synthesized using the chemical reduction method outlined by Singh *et al*., (2018). The prepared ZnONP suspensions were sonicated for an hour at 37°C in an ultrasonic water bath (Elmaonic S30H) to ensure proper dispersion.

Characteristics of ZnONPs:

ZnONPs with a size range of >30 nm and <50 nm were procured from NANO-FAB Co. (6 October, Egypt). The properties of these ZnONPs, as supplied by the manufacturer, included a purity of 99.5%, an average particle size of 30–50 nm, nearly spherical morphology, milky white color, specific surface area of 70 m^2/g, single crystal structure, and a true density of 5.5 g/cm^3.

Salinity Selection and Regeneration:

After 8 weeks on callus induction media, the most effective callus production medium (ZM3) was chosen. The calli from this medium were separated and transferred to fresh ZM3 media supplemented with varying concentrations of sodium chloride (NaCl) (0, 2500, 5000, and 10000 mg/l), labeled (Free salt Zinc Media) FZM3, (2500mg/l NaCl zinc media) S1ZM3, (5000 mg/l NaCl zinc media) S2ZM3, and (10000 mg/l NaCl zinc media) S3ZM3, respectively. This step aimed to subject the calli to shock selection for an additional 2 weeks to enhance proliferation and adaptability to salinity stress. The surviving calli were assessed for performance based on factors like color and fresh weight, with salt-adapted callus compared to control callus. Subsequently, plantlets were regenerated from the selected embryogenic calli by transferring them to magenta boxes containing MS-based regeneration media (RM) with 2 mg/ l 2,4-D, 1.88 gm /l Myo-inositol, and 3mg /l BAP. The pH was maintained at 5.8.

Except for S3ZM3, which failed to yield plantlets, regenerated plantlets from all treatments were transferred to a greenhouse with an aquarium containing Hoagland solution. Thereafter, they were transplanted to a soil medium composed of a mixture of 1/3 soil, 1/3 sand, and 1/3 peat moss for further growth.

The effect of salinity stress on the chemical composition of the obtained plants.

Total Leaf Chlorophyll Content:

Fresh fully expanded leaves were used to assess leaf chlorophyll content using the device of Minolta SPAD-502 Chlorophyll Meter, Minolta Co. Ltd, Japan. SPAD readings were taken at three positions on the leaf blade (top, left, and right of the middle vine). The Minolta SPAD-502 Chlorophyll Meter (Minolta Co. Ltd, Japan) was employed for readings. Average leaf chlorophyll content was calculated from the readings.

Percentage of Root Total Carbohydrate (mg/100 g dry weight):

Dried roots were ground into a fine powder using a stainless-steel Wiley mill. The determination of total carbohydrates in the roots was carried out using the spectrophotometric method described by Dubois *et al*., (1956).

Free Proline (mg/g dry weight):

Free proline content was measured calorimetrically in dry leaf samples using the method outlined by Zuniga *et al*., (1989).

Molecular Analysis:

Genomic DNA extraction was performed from callus and youngest fresh leaves of each treatment The CTAB method, with modifications based on Roger and Bendich (1985), was used for DNA isolation. Samples included an untreated control plant (S1), a control obtained from seeds (S2), and five representative samples for each salinity treatment (S3 to S7 for S1ZM3 plants, S8 to S12 for S2ZM3 plants, and S13 to S17 for S3ZM3 semi-dead callus). PCR amplification was carried out using a Biometra thermal cycler and 15 ISSR primers listed in Table (1). Prepared Promega Master Mix were used in a total volume of 25 µL (10 µL Master Mix, 10 µL dH2O, 3ul from 20 pmol of primer and 2 µL 10 ng genomic DNA).

The PCR profile consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at temperatures ranging from 44 to 53°C (depending on the primer) for 1.5 min, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min. Amplification products were first resolved on a 1.5 % agarose gel containing ethidium bromide in 1X TBE buffer, followed by resolution on 12% non-denaturing polyacrylamide gels. Molecular size standards (100 bp and 1Kb DNA ladder) were used for product visualization. PCR products were visualized and photographed using a Gel Documentation System (BIO-RAD).

Table 1. list of ISSR primers used in molecular evaluation of *Zea mays* L. plants.

#	Primers	Sequence 5' to 3'	G: C Content (%)	Tm (^{0}C)
1	ISSR 810	$(AG)^8TG$	50	54
2	ISSR 851	$(AC)^8CA$	44	45
3	ISSR 802	$(ACC)^6$	44	47
4	ISSR 852	$(AGC)^6$	50	48
5	ISSR 110	$(AC)^8G$	44	52
6	ISSR 114	$(CT)^8AG$	44	50
7	ISSR 115	$(TG)^8AA$	47	52
8	ISSR 125	$(GAG)^5AT$	50	54
9	ISSR 129	$(CT)^8GC$	53	47
10	ISSR 812	$G(ACAG)^3ACA$	44	47
11	ISSR 844	$(CGT)(ACT)(CGT)(GA)^5$	44	48
12	ISSR 885	$(AGT)(CGT)(AGT)(AC)^5$	47	53
13	ISSR 894	$(ACT)(ACG)(ACT)(TG)^5T$	44	46
14	ISSR 833	$(ACG)^4GAC$	52	54
15	ISSR 144	$(AGG)^6$	46	50

Statistical analysis

Amplified products for ISSR markers were visually examined for each primer or each primer combination. Also, Gel Documentation System (Gel-Doc 2000, with Diversity Database Fingerprinting Software, version 2.1, Bio-Rad Laboratories, Hercules, California, USA) was used for gel analysis, scoring, data handling, cluster analysis and construction of dendrograms. To determine the genetic relationships between control and different treatments.

RESULTS AND DISCUSSION

Callus initiation and somatic embryogenesis:

In this study focusing on callus initiation and somatic embryogenesis, 800 immature embryos from the GZ650 genotype were subjected to experimentation involving four distinct culture media: CM, ZM1, ZM2, and ZM3. The initial stages of culturing witnessed rapid growth of the scutellum, marked by its increased size, opaqueness, and early signs of peripheral cell proliferation. Following a two-week period, successful somatic embryogenesis was achieved from the scutellum side of immature embryos across almost all studied experiments. Embryogenic calli, distinguishable by their yellowish-white hue, demonstrated robust survival across multiple subcultures. Non-embryogenic calli were excluded from subsequent subculturing. Notably, the most effective callus induction, at 74.98 gm per 200 embryos with a mean of 0.37 gm per embryo, was observed in ZM3 medium containing 4000 ppm ZnONPs. Conversely, the least effective callus induction, at 31.76 gm per 200 embryos with a mean of 0.15 gm per embryo, was associated with CM medium, which lacked ZnONPs. The number of embryogenic callus pieces ranged from 86 in the control medium to 179 in the high-concentration ZnONPs medium (ZM3), as illustrated in Table 2. The presence of ZnONPs in the induction medium consistently outperformed the control medium in promoting embryogenic callus formation. The composition of ZM1, ZM2, and particularly ZM3 exhibited significant influence, with ZM3 resulting in 89.5% embryogenic calli compared to the control's 43%, as shown in Figure 1 (A, B, C, D, and E**).** These ZM3-derived embryogenic calli were subsequently used as the foundation for salinity experiments. This study emphasizes the influence of ZnONP concentrations on various aspects of maize growth and function, including antioxidants, DNA, protein synthesis, and molecular processes. It's important to acknowledge that the interactions between nanoparticles and plants are still a topic of ongoing research, revealing potential advantages and drawbacks depending on concentration and environmental factors.

Table 2. callus induction Frequency of the GZ650 maize inbred line at different concentrations of ZnONPs.

Treatment	No. of embryos	Total Callus weight gm after		Average No. of embryogenic calli	status	color
		4w	8w			
CM (0PPM)	200	22.01	31.76	86	compact	white
ZM1(1000ppm)	200	29.3	37.97	93	compact	white
ZM2 (2000ppm)	200	41.88	59.07	144	friable	Yellowish -white
ZM3(4000ppm)	200	49.15	74.98	179	friable	Yellowish -white

The findings of this investigation emphasize the crucial role of ZnONPs in promoting the initiation of a friable embryogenic callus. These outcomes align with previous research by Aghdaei *et al*., (2012) and Hassan *et al*., (2019), which also highlighted the beneficial effects of nanoparticles (NPs) in enhancing the growth and development of cultured olive explants. Notably, Alsuwayyid *et al*., (2022) discovered that ZnONPs exhibited dual effects on seedlings, displaying positive impacts at lower concentrations and negative effects at higher concentrations, resulting in the accumulation of these nanoparticles and increased Zn2+ levels. A similar positive influence of nanoparticles on plant growth has been demonstrated by EL-Kady *et al*., (2017), who explored the impact of silicon dioxide nanoparticles on banana plant rooting rates, photosynthetic pigments, and morphological traits. This supports the broader idea that nanoparticles can effectively enhance micropropagation and overall plant development, which has been observed in various plant species (Sarmast *et al*., 2015; Hegazi *et al*., 2018). The impact of ZnONP concentrations on maize morphology and physiology is highlighted in this study. These nanoparticles' effects extend to antioxidants, DNA, protein synthesis, and molecular functioning, as indicated by previous investigations (Karimizarchi *et al*., 2014; Kim *et al*., 2018; Goswami *et al*., 2019; Rajput *et al*., 2020; Wang *et al*., 2018). It is worth noting that the interactions between nanoparticles and plants remain a subject of active research, with studies consistently unveiling both their potential benefits and potential negative impacts based on concentration and context.

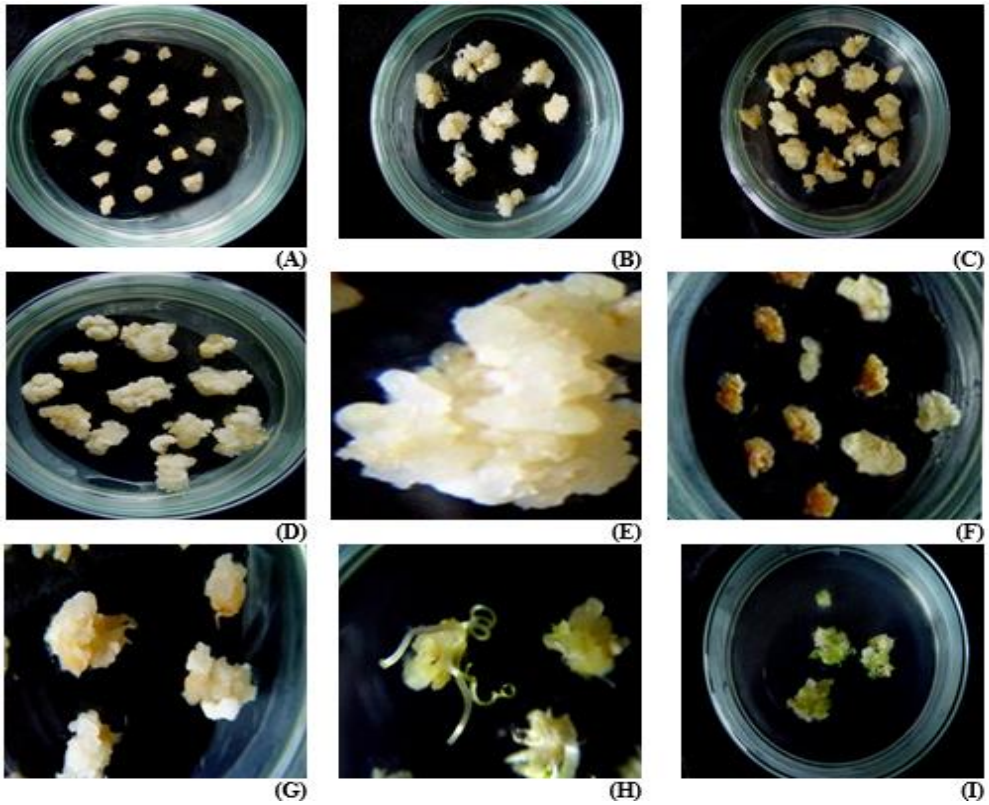

Fig. 1. GZ650 maize immature embryos: (A) Induced calli on CM media. (B and C) somatic embryogenesis on ZM1 and ZM2 media. (D and E) Type II callus on ZM3 medium. (F and G) 2 weeks of incubated callus on selection ZM3 media containing 2500 and 10000 mg/l NaCL, respectively. (H and I) Callus proliferation on regeneration media and early shoot induction.

Effect of salinity stress on callus performance

Also, the change in callus color before and after selecting salinity was updated, as it was clear that the FSZM3 callus kept its creamy white color, while salinity-exposed callus changed to yellowish brown, as well as reddish yellow in moderate concentrations of salinity (2500 and 5000 ppm), and finally changed to blackish brown in high concentration of salinity (10000 ppm) Figure 1 (F and G). Additionally, the texture of the callus was converted from the friable form to compact, intermediate (compact and friable), or watery configurations with an increase in salinity.

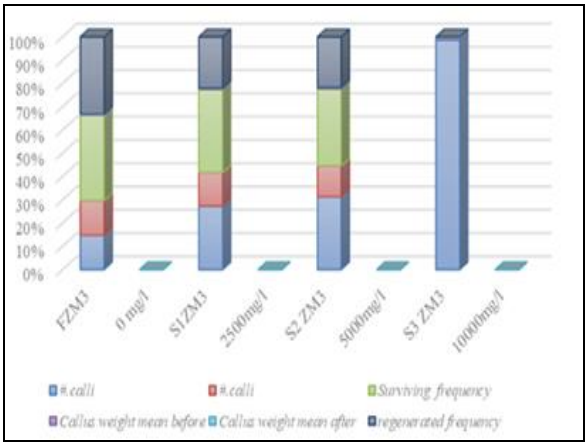

Fig. 2. The number of calli in GZ650 maize, callus weight before and after salinity treatments, and their regeneration frequency.

Analyzing the data presented in Figure (2)**,** it becomes evident that calli cultivated in FSZM3 (Free Salt Zinc Oxide Media) displayed a 100% survival rate upon transfer to regeneration media with fast proliferation Fig 1 (H and I), yielding a significant number of plantlets with a high regeneration frequency of 90% as compared with different concentration salinity media, which produced 32.4% and 27.89% with S1ZM3 and S2ZM3, respectively, which reflect the effect of salinity as shown in Figure 3 (A, B, and C)**.** In contrast, all calli cultured in S3ZM3 (NaCl media with a concentration of 10000 mg/l) succumbed, failing to generate any regenerated plants, thus exhibiting a regeneration frequency of 0.0%. As well as the emergence of some abnormal plantlets with a red-greenish color, especially on S2ZM3 media (Fig. 3D)**.** Furthermore, calli cultivated in FSZM3 media exhibited a substantial increase in mean weight. Conversely, an observable reduction in callus weight was apparent across different salinity concentrations, concurrently influencing callus texture and color transition.

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on S2ZM3 media Figure 3(E)**.** Furthermore, calli cultivated in FSZM3 media exhibited a substantial increase in mean weight. Conversely, an observable reduction in callus weight was apparent across different salinity concentrations, concurrently influencing callus texture and color transition. Therefore, all shooted calli were transferred after 2 weeks to the freshly regenerated media for 2 weeks again for plantlet development and to develop roots Figure 3 (F, G, H and I)**.** Regenerated plantlets were acclimatized to the Hoagland solution for 3–4 days. Plantlets formed axial roots and 2-3 leaves and were transferred to pots and later transplanted to soil. Most plants were morphologically normal, similar to the original line, reaching full maturity. These findings corroborate the conclusions drawn by James *et al*., (2011), who emphasized that heightened NaCl concentrations within tissue culture media disrupt membrane stability through the displacement of K+ and Ca2+ ions. Consequently, the observed water loss and compromised membrane integrity impede the callus' ability to absorb essential ions, leading to reduced water and nutrient uptake. Similarly, Turan *et al*., (2010) elucidated that elevated salinity levels detrimentally affect callus capacity to assimilate crucial ions such as N, Ca, K, P, Fe, and Zn, further contributing to the compromised growth and development observed in this investigation.

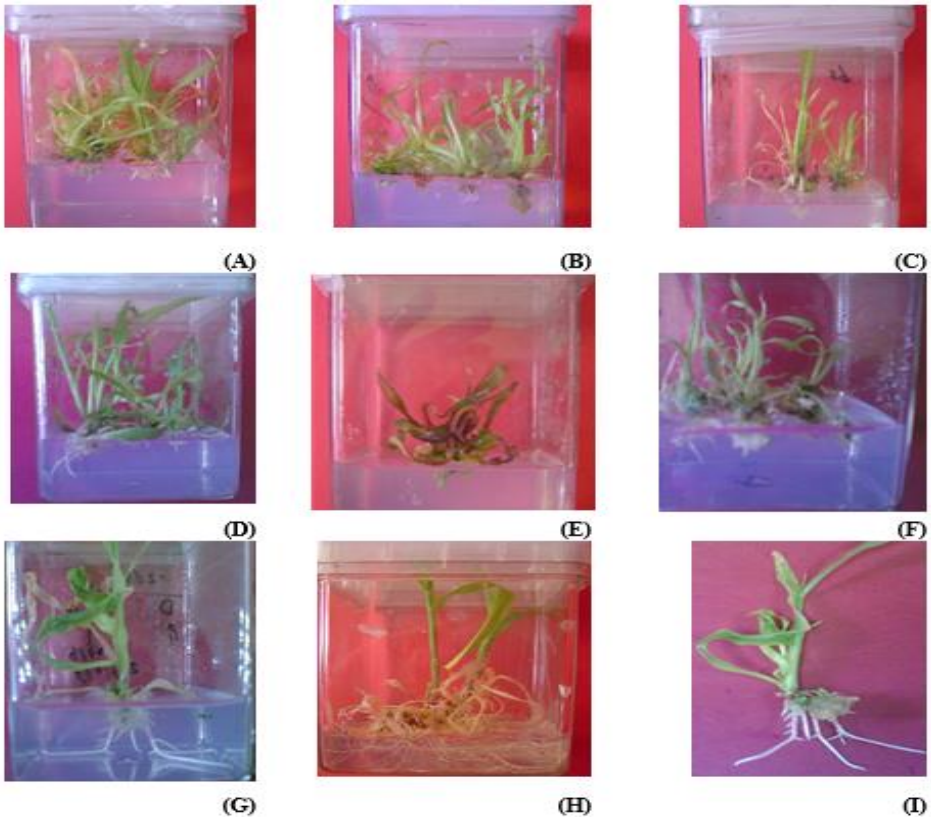

Fig. 3. Maize regeneration: (A) CM media plantlets after transferred to regeneration media; (B, C, and D) ZM1, ZM2, and ZM3 plantlets on regeneration media, respectively. (E) Regenerated maize plantlets with red, green color on regeneration media. (F, G, and H) Regenerated maize plantlets after 4 weeks on regeneration media. (I) maize plantlets before transferred to acclimatization

The chemical composition of GZ650 maize plants as affected by salinity stress.

Maize production losses due to drought and salinity prominently affect economies and the livelihoods of millions of people, given the global and regional importance of maize and its pronounced susceptibility to these stress factors. Climate change and accelerating competition for irrigation water are expected to further increase the need for adaptive strategies. There is vast evidence for genetic approaches being able to significantly improve the drought and salinity tolerance of maize, according to Marianne and Jose-Luis (2007).

Total leaf chlorophyll content:

The investigation focused on the total leaf chlorophyll content, revealing distinct differences among the studied plant groups, as demonstrated in Figure 4. Notably, FSZM3 plants cultivated in media containing free salts exhibited the highest photosynthetic pigment values, followed by plants obtained from GZ650 line seeds. Conversely, S2ZM3 plants, grown in media supplemented with 5000 mg/l NaCl, displayed the lowest chlorophyll content. Under salinity stress, a significant reduction in total leaf chlorophyll content was observed across all plant groups. Interestingly, plants treated with 5000 ppm ZnONPs displayed improved chlorophyll content compared to control seed plants. The results highlight the sensitivity of chlorophyll content to salinity stress and its modulation by ZnONPs treatment. The variations in chlorophyll levels among plant groups suggest diverse physiological responses to stress conditions. The decrease in chlorophyll content under salinity stress aligns with Santos *et al*., (2009), who observed reduced stomatal conductance and net photosynthetic rate due to a water deficit. Terzi *et al*., (2010) further documented the susceptibility of chlorophyll content to drought stress, leading to significant decreases in chlorophyll a, chlorophyll b, and total chlorophyll contents. The insights from Yu *et al*., (2009)

underscore the complex mechanisms at play, as they noted water stress-induced inhibition of photosynthesis via diminished ribulose-1,5-bisphosphate (RuBP) supply, possibly linked to low ATP synthesis. The enhancement of growth and chlorophyll content in wheat through ZnONPs seed priming, as demonstrated by Munir *et al*., (2018) and Rai-Kalal and Jajoo (2021), underscores the potential benefits of nanomaterials in stress mitigation.

Root total carbohydrate (mg/100 g dry weight).

Analyzing the data presented in Figure 4, it is evident that significant differences in root total carbohydrate (mg/100 g dry weight) were observed across the treatments. Particularly noteworthy is the substantially higher root total carbohydrate content in FSZM3 plants (grown in free salt media containing a high concentration of ZnONPs) compared to all other treatments. Conversely, a consistent reduction in total carbohydrate content was evident with increasing levels of salinity stress. This decline could be attributed to the conversion of carbohydrates into simple sugars utilized for respiration, a metabolic shift often induced by stress conditions. A similar phenomenon was noted by Saleh (2007) in mung bean under chilling stress conditions. The accumulation of soluble carbohydrates in plants has been widely recognized as a critical response to salinity or drought stress, serving as an important strategy for osmotic adjustment and maintenance of cell turgor (Ali *et al*., 2000; Ashraf, 2004). Under salt stress conditions, plants often increase their content of reducing and non-reducing sugars, along with the activity of sucrose phosphate synthase, as observed in numerous plant species (Parida and Das, 2005). Our study's findings corroborate these observations.

Free proline (mg/g dry weight).

In this study, the variations in proline content among different treatments were highlighted, as detailed in Table 4. The highest levels of free proline content (12.95 mg/g dry weight) were observed in S2ZM3 plants, while the lowest levels were found in FS ZM3 and seed Figure (4). This observation aligns with the notion that proline accumulation is a significant mechanism for plant resistance against various stress factors, such as drought (Naidu *et al*., 1992). The accumulation of proline, along with minimal disruptions in chloroplast structure, in tolerant genotypes. Thus, the buildup of free proline is considered an important indicator for selecting stress-tolerant plants (Flores, 1997). Comparing the findings, the higher levels of free proline content in ZnONPs-treated plants compared to other treatments suggest a potential role for proline as an adaptive mechanism in response to water and salinity stresses. This contrasts with the results of Maiti *et al*., (2000), who observed a general increase in free proline under escalating drought stress. Similarly, Revilla and Canal (1999) documented increased proline levels in olive calli subjected to salinity stress. The proline accumulation during drought stress is proposed to function as a scavenger of reactive oxygen species (ROS), contributing to enhanced adaptation and growth under such conditions (Turkan and Demiral, 2009). The accumulation of proline is a crucial indicator of drought stress tolerance in higher plants (Ashraf and Iram, 2005). Proline's role in overcoming osmotic stress due to water loss has been suggested (Caballero *et al*., 2005). It's a non-protein amino acid formed in tissues exposed to water stress and is metabolized upon rehydration following drought (Singh *et al*., 2000). Apart from its Osmo protectant function, proline acts as a sink for energy, regulating redox potentials, and scavenging hydroxyl radicals (Sharma and Dietz, 2006). Additionally, proline protects macromolecules from denaturation and reduces cellular acidity (Kishor *et al*., 2005). In terms of salt stress tolerance, while Vendruscolo *et al*., (2007) suggested that proline might enhance the antioxidant system and not merely serve as an osmotic adjuster for conferring salt stress tolerance to wheat plants, the current study primarily emphasizes proline's role as an osmoregulatory in maize cells under water and salinity stresses. Overall, the findings of this study align with previous research in highlighting proline's significance in stress tolerance, particularly drought and osmotic stresses, while also shedding light on potential variations in its roles under different stress conditions such as salinity.

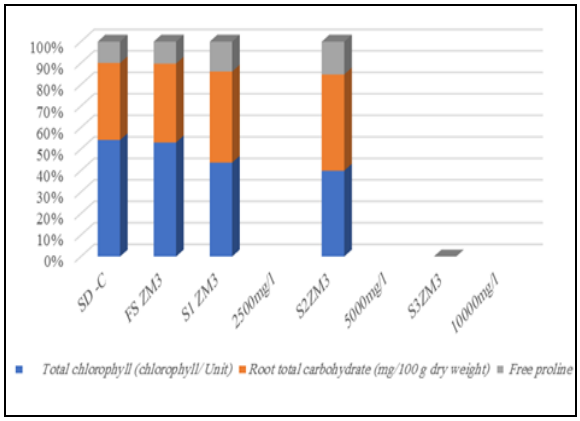

Fig. 4. Chemical composition of control and salinity treated maize plants

Genetic Variation Analysis and Genetic Relationship Among Maize Samples Using ISSR Markers:

Genetic diversity assessment plays a pivotal role in understanding the variations present within plant populations, and this study utilized the power of 15 ISSR primers to analyze the genetic variation among the obtained plant and callus samples. These primers, selected for their ability to detect polymorphism, were employed to estimate the genetic diversity among different treatments and compare them with the control group. The chosen ISSR primers generated multiple band profiles, amplifying a range of DNA fragments (7 to 12) with an average of 9.53 bands per primer. Among these fragments, 1.23 on average were polymorphic, indicating variations in the genetic makeup. A maximum of 12 fragments were amplified by ISSR primer 802, while a minimum of 7 fragments were observed with ISSR primer 833. In total, across the fifteen primers, 143 reproducible fragments were amplified, of which 19 were polymorphic. This translated to a polymorphism level of 13.28%, underscoring the high genetic diversity within the studied samples, as shown in Table 3. The sizes of the amplified fragments varied with the primers, ranging from 50 to 1300 base pairs Figure (5 A and B). To assess genetic relatedness, genetic distances were calculated between all treated samples, yielding a similarity matrix. Utilizing the UPGMA method, dendrograms were constructed, providing a visual representation of genetic relationships. Genetic similarity among the 17 maize samples ranged from 66% to 100%, with an average of 83%. This finding indicated a relatively

low level of polymorphism at the DNA level, which aligns with the focus on a single genotype under salinity stress conditions using ZnONPs media. Notably, samples from closely related treatments exhibited higher similarity levels, as seen in the 100% similarity between S3 and (S6-S7), originating from the same salinity treatment. Conversely, the lowest genetic similarity (66%) was observed between S5 and S17, differing in their NaCL concentrations. Moreover, a range of 75% to 81% genetic similarity was observed between high salinity-focused plants (S12 to S17) and control plants (S1 and S2), reflecting significant genetic changes due to high salinity treatments, as illustrated in Table 4.

This genetic divergence due to salinity stress findings was supported by previous studies by Suryanto (2003), Ruwaida (2009), Hefny *et al*., (2017), and Serag (2021), which highlighted genetic alterations caused by shifts in DNA nucleotide composition under stress. Additionally, in the context of maize drought tolerance evaluation, Badr and Breggemann (2020) employed similar indices based on seedling traits under stress conditions versus controls.

Comparative studies revealed lower genetic diversity in other maize populations. For instance, ISSR primers led to only 36.46% polymorphism in CIMMYT maize populations (Berilli *et al*., 2011) and 29 polymorphic fragments across 20 maize genotypes using SCoT markers (Vivodík *et al*., 2017).

The genetic relationships among the 17 maize samples were adeptly depicted through UPGMA-based dendrograms. The clustering process successfully segregated the samples into two primary clusters, primarily distinguished by their NaCl concentration treatments: one encompassing the control group and another containing samples treated with NaCl concentrations of 2500 and 5000 ppm. Notably, samples treated with a 1000 ppm concentration formed a distinct subgroup. This pattern was consistently corroborated by both Principal Component Analysis (PCA) and the heatmap analysis in Figure 6.

Table 3. ISSR Marker Analysis Results for the 17 Maize Samples, including Polymorphism Levels, Total Band Count, Monomorphic Bands, Polymorphic Bands, and Percentage of Polymorphism.

No.	Primers	Total number of bands	Monomorphic bands	Polymorphic bands	% of polymorphism
1	ISSR 810	11	9	2	18.18
2	ISSR 851	10	10	0	0
3	ISSR 802	12	9	3	25
4	ISSR 852	10	9	1	10
5	ISSR 110	9	9	0	0
6	ISSR 114	10	10	0	0
7	ISSR 115	8	8	0	0
8	ISSR 125	9	9	0	0
9	ISSR 129	10	8	2	20
10	ISSR 812	9	7	2	22
11	ISSR 844	8	8	0	0
12	ISSR 885	11	8	3	27
13	ISSR 894	10	9	1	10
14	ISSR 833	7	7	0	0
15	ISSR 144	9	9	0	0
Total		143.0	124	19.0	132
Average		9.53	8.26	1.26	8.8

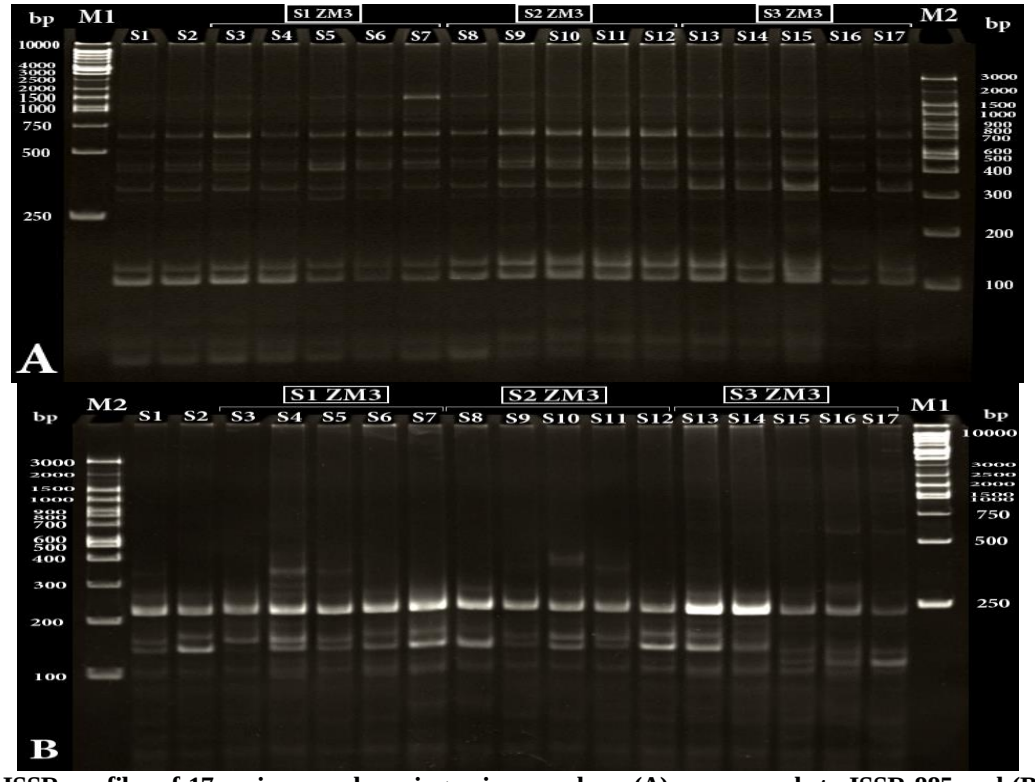

Figure 5. ISSR profiles of 17 maize samples using primers, where (A) corresponds to ISSR 885 and (B) to ISSR 125. Among the lanes, S1 and S2 depict the untreated control and seed plant, respectively. S3 to S7 pertain to S1ZM3 plant samples, S8 to S12 correspond to S2ZM3 plant representatives, and S13 to S17 represent samples from semi-dead S3ZM3 callus. Notably, DNA molecular weight standards (M1 and M2) were incorporated using 100 bp and 1 kb DNA ladder markers.

Table 4. Genetic dissimilarity matrix within and among 17th maize samples as computed according to Dice's similarity coefficient from ISSR generated data.

	S1	S2	S1ZM31	S1ZM32	S1ZM33	S1ZM34	S1ZM35	S2ZM31	S2ZM32	S2ZM33	S2ZM34	S2ZM35	S3ZM31	S3ZM32	S3ZM33	S3ZM34	S3ZM35
S1	0	0	0.16	0.192	0.192	0.16	0.16	0.188	0.208	0.22	0.22	0.17	0.188	0.208	0.208	0.224	0.245
S2		0	0.16	0.192	0.192	0.16	0.16	0.188	0.208	0.22	0.22	0.17	0.188	0.208	0.208	0.224	0.245
S1ZM31			0	0.041	0.041	0	0	0.146	0.167	0.25	0.216	0.204	0.255	0.275	0.275	0.288	0.308
S1ZM32				0	0	0.041	0.041	0.18	0.2	0.245	0.212	0.235	0.283	0.302	0.302	0.315	0.333
S1ZM33					0	0.041	0.041	0.18	0.2	0.245	0.212	0.235	0.283	0.302	0.302	0.315	0.333
S1ZM34						0	0	0.146	0.167	0.25	0.216	0.204	0.255	0.275	0.275	0.288	0.308
S1ZM35							0	0.146	0.167	0.25	0.216	0.204	0.255	0.275	0.275	0.288	0.308
S2ZM31								0	0.024	0.13	0.089	0.07	0.25	0.271	0.271	0.286	0.306
S2ZM32									0	0.152	0.111	0.093	0.271	0.255	0.292	0.271	0.292
S2ZM33										0	0.044	0.068	0.245	0.265	0.265	0.28	0.3
S2ZM34											0	0.068	0.245	0.265	0.265	0.28	0.3
S2ZM35												0	0.196	0.217	0.217	0.234	0.255
S3ZM31													0	0.024	0.024	0.047	0.07
S3ZM32														0	0.048	0.024	0.048
S3ZM33															0	0.07	0.048
S3ZM34																0	0.024
S3ZM35																	0

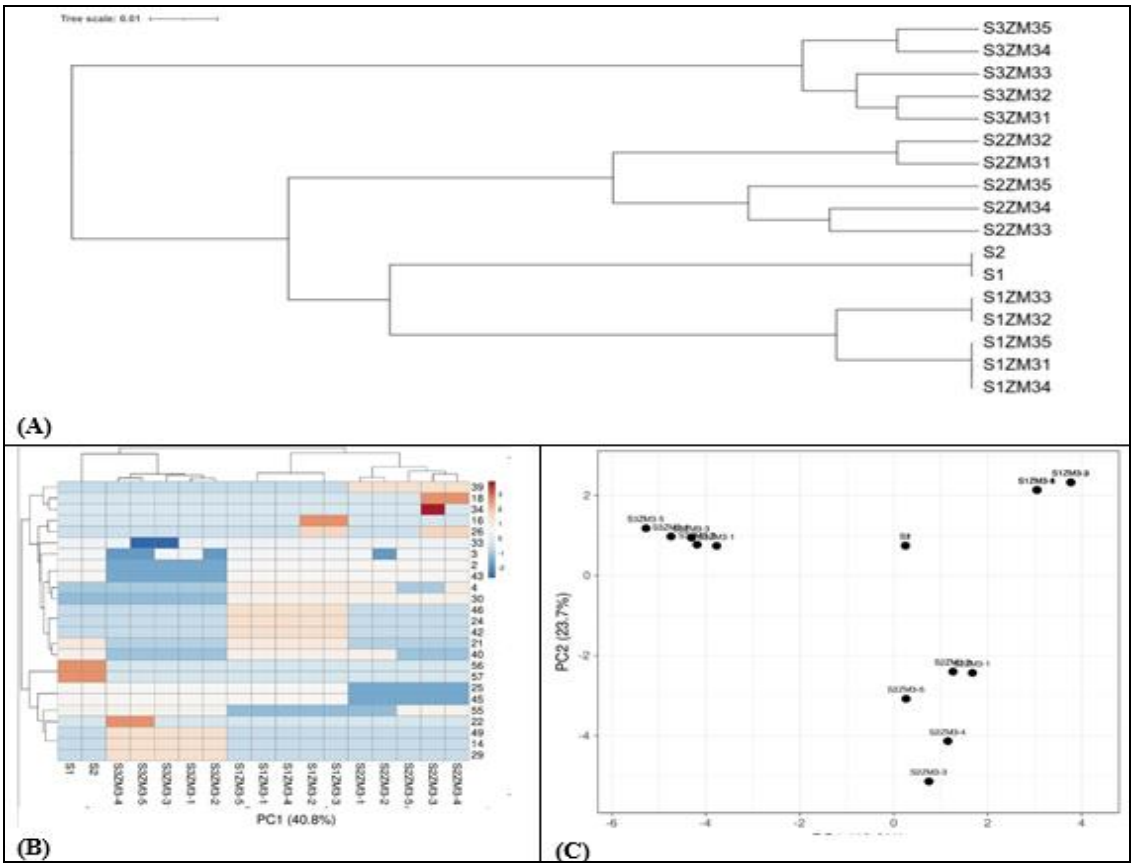

Figure 6. (A): Dendrogram for the 17th maize samples constructed from the ISSRs generated data using the UPGMA method and similarity matrices computed according to DSC's. (B): the Heatmap (C): PCA.

Subclusters further categorized the samples based on control and different salinity treatments. These findings resonated with Carvalho *et al*., (2002), emphasizing genetic variability in Brazilian maize landraces, and Junior *et al*., (2011), advocating for the inclusion of more distinct genotypes in breeding programs.

In conclusion, ISSR markers proved to be powerful tools for assessing genetic diversity and relationships among maize samples under varied salinity treatments. The study highlighted the impact of salinity stress on genetic makeup and demonstrated the potential for selecting genotypes with distinct traits for future breeding programs, in line with recommendations from Muhammad *et al*., (2017).

In conclusion, this study has successfully established a reliable regeneration protocol for GZ650 Egyptian maize inbred lines, leveraging immature embryo explants and ZnONPs-infused callus induction medium. Notably, the inclusion of 4000 ppm ZnONPs yielded the most promising results in augmenting the formation of embryogenic calli. When exposed to varying salinity levels, calli exhibited diverse reactions, with 2500 and 5000 ppm NaCl concentrations fostering robust calli that produced adaptable plantlets. Chemical analysis revealed heightened levels of chlorophyll, proline, and total carbohydrates in ZnONPs-treated plants, suggesting their role in enhancing plant resilience against salinity stress. Furthermore, the ISSR data underscored the substantial genetic variations induced by high-salinity treatments. In summary, this research sheds light on the potential of ZnONPs to facilitate embryogenic callus development and

enhance plant resistance to salinity stress. These findings not only contribute to the advancement of plant tissue culture techniques but also offer insights into strategies for enhancing crop resilience in challenging environments.

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تحفيز بدء الكالس ومقاومة الملوحة في الذرة المصرية (*Zea mays* L.) GZ650 بإستخدام جزيئات أكسيد الزنك النانوية

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الملخص

تم دراسه بدء الكالس والتكوين الجنيني الجسدي داخل الطراز الوراثي للذره (GZ650)، مع التركيز علي تأثير البيئه المتميزه والجسيمات النانويه لأكسيد الزنك (ZnONPs). تضمنت التجربه ٨٠٠ جنينًا في أربعه بيئات مختلفه (CM, ZM1, ZM2, ZM3).تم تكوين جنين جسدي ناجح، حيث تحتوي البيئه ZM3 علي ٤٠٠٠ جزء في المليون من ZnONPs مما يدل علي تكوين الكالس الأمثل. تفوقت ZnONPs باستمرار في التحكم في تعزيز تكوين الكالس الجنيني، ولا سيما مع ZM3 والذي انتج ٨٩,٥٪ من الكالس الجنيني مقارنة بنسبة ٤٣٪ للكنترول. كشفت تجارب الملوحة على الكالس الجنيني المشتق من ZM3 عن تأثيرات على أداء الكالس. أظهر تحليل التنوع الوراثي باستخدام (ISSR)التشابه والاختلاف بين عينات الذرة تحت تأثير الملوحة. تؤكد هذه الدراسه على قدره ZnONPs في تسهيل تطوير الكالس وتعزيز مرونة النبات ضد إجهاد الملوحة والتقييم الوراثي لإستجابه ZnONP والملوحه.