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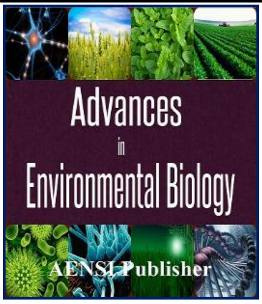

Using Different Growth Regulators in Wheat to Overcome Negative Effects of Drought Stress as One of Climate Change Impacts and Evaluation of Genetic Variation Using ISSR

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ABSTRACT

Drought is a worldwide problem, constraining global crop production seriously and recent global climate change has made this situation more serious. This study was planned to evaluate the potential of kinetin and salicylic acid as a growth regulator to overcome the negative effects of stimulated drought stress on two wheat (*Triticum aestivum* L.) cultivars (Gimeza 9 and Sakha 93). Two experiments (pots and field) were carried out during two successive seasons. Water deficit was imposed by withholding water after milking stage to season end. Yield and quality traits were evaluated at end time. Results showed that drought stress caused an increase in proline, total carbohydrates, catalase and peroxidase enzyme while decrease grain yield, protein (gluten) and macronutrient content (N, P, and P) of both wheat cultivars. The impact of drought stress is more damaging in the Gimeza 9 cultivar compared with Sakha 93 cultivar. Kinetin and salicylic acid treatments reduced the damaging action of drought stress on wheat plants. Both kinetin and salicylic acid treatments increased wheat grain yield, biochemical characters and antioxidant enzyme content under control and stress conditions. These effects were more pronounced with kinetin treatment. The impact of kinetin and salicylic acid used to overcome drought damage was more pronounced on the Sakha 93 cultivar than Gemiza 9 cultivar. Moreover, using ISSR was robust and detects minor variations fast, reliable and efficiently for both tested wheat cultivars treated to adopt drought stress.

KEYWORDS: Stress, Wheat, Stress tolerance, Growth regulators, Kinetin, Salicylic acid, Antioxidant enzymes, Proline; ISSR

INTRODUCTION

Drought is a worldwide problem, constraining global crop production seriously and recent global climate change has made this situation more serious. Wheat is one of the most important cereal crops grown in the world which plays a key role in the economic activity. In Egypt, the cultivated area was around three million feddan yearly. Increasing the cultivated area of wheat should be done in the reclaimed land due to the limited areas of the Nile Valley and the competition of the main crops. Wheat should be irrigated when 50–55% of the available soil water is depleted in the root zone [1]. Water stress (drought) is the most important factor that affecting the productivity of wheat. Across plant species, drought imposes various physiological and biochemical limitations and adverse effects [2, 3]. Exposing plants to water stress adversely affect plant growth and productivity [4]. The decrease in soil water potential causes alteration in minerals uptake by plant roots and reduction in leaf expansion under drought or salinity stress conditions [5]. During grain development of wheat, appropriate soil water status is of key importance for accumulation assimilates in grains and thus formation of grain yield and quality [6]. Egypt presents a typical example of the drought problem faced in some arid districts. There is a critical need to balance water availability, water requirements and water consumption. Thus, water conserving is becoming a decisive consideration for agriculture. Exploiting and increasing production in these areas are necessary to bridge the gap between production and consumption of wheat. There are different approaches to mitigate the drought hazards, which include the development of stress tolerant plants by selection of stress resistant varieties [7], *in vitro* selection, use of plant growth hormones (ABA, GA_3, K, SA), antioxidants (ascorbic acid, H_2O_2) and osmoprotectants as foliar application and seed treatment [8]. Among growth regulators, cytokinins have critical role for the promotion of cell division, chlorophyll biosynthesis and modification in apical dominance in plants [9]. Cytokinin application under abiotic stressful conditions can delay the leaf senescence directly by scavenging free radicals [10]. Salicylic acid is considered to be a potent plant hormone [11, 12]. Salicylic acid is a plant growth regulator that is part of a signaling pathway induced by several biotic and abiotic stresses [13]. Exogenous application of SA has been shown to induce plant stress tolerance. For example, treatment of common bean and tomato plants with salicylic acid increased their drought tolerance. Exogenous application of salicylic acid also has been reported to modulate activities of intracellular antioxidant enzymes SOD and POD and increase plant tolerance to environmental stresses [14, 15, 16]. This study was planned to appraise the potential of both kinetin (K) and salicylic acid (SA) as a growth regulator to overcome negative effects of environmental drought stress in wheat and evaluate the genetic variation using ISSR.

MATERIALS AND METHODS

Two experiments (pots and field) were carried out during two successive seasons (2014 and 2015) at the Greenhouse and Experimental Farm, Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt, to evaluate the potential of kinetin and salicylic acid as growth regulators to overcome negative effects of stimulated drought stress in wheat. Water deficit (drought stress) was imposed by withholding water after milking stage to season end. Grains of two wheat (*Triticum aestivum* L.) cultivars (sensitive Gimeza 9 and resistant Sakha 93) were used in this study.

Greenhouse experiment:

A pot experiment was carried out during season 2014 to test the effect of kinetin and salicylic acid on wheat plant growth under stimulated drought stress conditions. Grains of two wheat cultivars were sown in each pot (20 cm diameter) with three replicates, each pot contained 15 kg soil. Experiment was designed in complete randomize design. Plant dry weight at vegetative growth period was recorded. Soils were fertilized with N, P and K with recommended rates.

Field experiment:

The field experiment was carried out during season 2015 to verify the potential of Kinetin (K) and salicylic acid (SA) in improving the growth, yield and chemical composition of wheat plant under drought stress conditions. Field experiment consisted of $4m^2$ (2x2m) in area plots with three replicates for each treatment as well as control. The experimental layout was a split-split plot design based on randomized complete blocks. Physical and chemical characteristics of the studied soil before planting are presented in Table (1). Soil was fertilized with N, P and K with recommended rates. Three times of foliar application was carried out, the first time was after 90 days from sowing (booting stage) and the second time was after 105 days from sowing (heading stage) and the third time was after 120 days from sowing (milking stage). On harvest, plants from each group were collected to determine growth characters, yield and yield components as well as the biochemical changes in the yielded grains.

The two experiments consisted of Six Treatments:

T1: No-stress (control)
T2: No-stress + 10 mg/l K
T3: No-stress + 100 mg/l SA
T4: Drought stress
T5: Drought stress + 10 mg/l K
T6: Drought stress + 100 mg/l SA

Analytical methods:

Physical and chemical characteristics of the studied soil were determined according to Page *et al*. [17]. In plant sample, phosphorus content was determined by vanadomolybdate yellow method spectrophotometrically and K by flame photometer [18]. Total nitrogen was determined by micro-Kjeldahl method according to AOAC [19]. Crude lipid was determined using the Soxhlet extraction method according to AOAC [20]. Total carbohydrate was extracted according to Smith, *et al*. [21] and determined using spectrophotometer according to Murphy [22]. Concentration of free proline was colorimetrically measured in wheat grain using ninhydrin reagent by spectrophotometer. Gluten content was determined by employing the procedure of Paul [23]. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (ε = 40mM−1 cm−1) according to the method of Aebi, [24]. The reaction mixture contained 50mM K-phosphate buffer (pH 7.0), 33mM H_2O_2 and enzyme extract. Peroxidase activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol (ε = 26.6mM^{-1} cm^{-1}) according to the method of Polle *et al*. [25]. The reaction mixture contained 100mM K-phosphate buffer (pH 7.0), 20.1mM guaiacol, 10 mM H_2O_2 and enzyme extract. The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 5 min. All determinations were performed in triplicate and data represented on dry weight basis as mean values ± standard deviations. At the end of season all plants were harvested and collected per plot and yield per feddan were calculated.

Table 1: Physical and chemical properties of the tested soil

Physical	Value
Coarse sand (%)	6.3
Fine sand (%)	20.9
Silt (%)	38.3
Clay (%)	34.5
Texture soil	loamy clay
Chemical	
pH (1: 2.5, soil suspension)	7.78
Organic matter (%)	1.11
ECe dS m^{-1}, soil paste	1.76
Soluble cations (me/L)	
Ca^{++}	7.0
Mg^{++}	3.8
Mg^{++}	5.1
K^{++}	1.5
Soluble anions (me/L)	
$CO_3^{=}$	-
HCO_3^{-}	4.1
Cl^{-}	7.1
$SO_4^{=}$	6.2

ISSR Marker Analysis:

Three ISSR primers were used in the present study. DNA was amplified according to the following protocol. Each PCR reaction mix of 25 µL contained the 30 ng template DNA, 5 µL of 5X PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 2.5 µL of the dNTPs mix, 30 pmol of ISSR primer, 1.0 U Taq DNA polymerase (Promega, WI, USA). The amplification was performed in a thermal cycler (Applied BioSystems, USA) programmed for initial denaturation of 5 min at 94°C; 35 cycles of 50 sec denaturation at 94°C, 50 Sec annealing at 50°C and 1.5 min extension at 72°C; and final elongation step at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide 0.5 µg/mL in TBE buffer for 2 hr at 100 V. After electrophoresis, the gels were observed under an UV-transilluminator, documented in Gel-Doc XR (Bio-Rad) and photographed. The size of the amplicons was determined using 100 bp DNA ladder plus.

Markers Data Analysis:

The generated/ amplified bands were scored visually. The bands were scored as present (1) or absent (0) to create the binary data set. To estimate the genetic similarity, Jaccard's coefficient was used. A dendrogram was generated by cluster analysis using the un-weighted pair group method of the arithmetic averages (UPGMA) using SPSS program V1.6. Support for clusters was evaluated by bootstapping analysis. One thousand permutation data sets were generated by re-sampling with replacement of characters within the combined 1/0 data matrix.

Statistical Analysis:

All data were statistically analyzed according to Snedecor and Cochran [26].

RESULT AND DISCUSSION

Drought is a notable factor that affects the growth and physiological characteristics of plants [27]. The present study was to evaluate the potential of kinetin and salicylic acid in improving wheat growth and yield under drought stress conditions.

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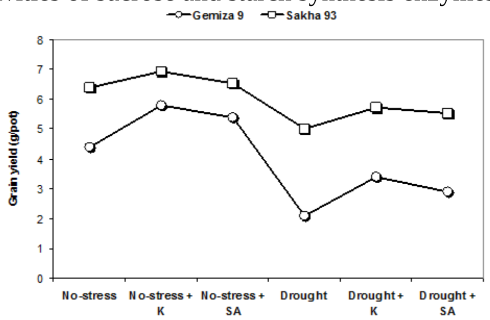
Growth and Yield:

Drought stress reduced the grain yield of both wheat cultivars. This effect was more marked with sensitive cultivar plants. The used K or SA improved the grain yield of both cultivars. K or SA treatments appeared to alleviate the effect of drought stress on wheat plants under either control or stress conditions. It was shown that there was no significant difference recorded between no stress treatment and both tested drought stress treatments with either K or SA as shown as in Fig. 1 and 2. Results recorded were approximately similar in both pot and field experiments. It was found that K treatment was more effective than SA treatment relatively. Drought-related reduction in yield and yield components of plants could be ascribed to stomatal closure in response to low soil water content, which decreased the intake of CO_2 and as a result, photosynthesis decreased. Drought reduces plant growth and development, leading to hampered flower production and grain filling and thus smaller and fewer grains. A reduction in grain filling occurs due to a reduction in the assimilate partitioning and activities of sucrose and starch synthesis enzymes.



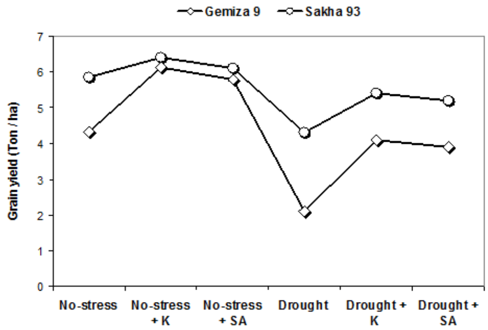


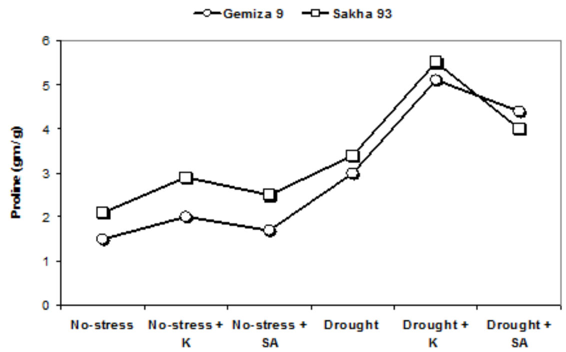
Fig. 1: Effect of Kinetin (K) or salicylic acid (SA) on grain yield of wheat under stress and non stress conditions for pot experiment

Fig. 2: Effect of Kinetin (K) or salicylic acid (SA) on grain yield of wheat under stress and non stress conditions under field experiment

Chemical composition:

Proline and total carbohydrate:

As compared to the control values (no-stress), drought stress caused significant increaments in proline and as well as total carbohydrate concentrations in both wheat cultivars as shown as in Fig 3 and 4, whereas, proline content in Sakha 93 was higher than in Gemiza 9. It was shown that both stress treatments (K and SA) scored high increaments in proline and total carbohydrate when compared with control treatment (no stress) and reached level of significance. Also, it is worth mentioning, that application of K or SA caused significant increase in proline and carbohydrate content in both tested cultivars. Plants treated with K scored higher readings in proline and carbohydrate contents than those treated with SA across the two tested cultivars under both control (no-stress) and stress conditions. These results are in agreement with those of Mostajeran and Rahimi-Eichi [28], who indicated that drought stress causes increases in proline and soluble carbohydrates concentrations. Proline and carbohydrates are major constituents of osmoregulation in the expanded leaves of many species [4]. Besides osmotic adjustment, other possible functions of proline include the protection of plasma membrane integrity, the prevention of protein denaturation, being a sink of energy or reducing power, being a source for carbon and nitrogen and acting as a hydroxyl radical scavenger [29]. The accumulation of carbohydrates in response to drought stress is also well documented [30].



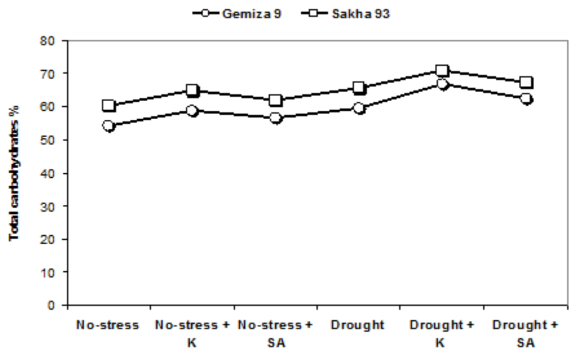


Fig. 3: Effect of Kinetin (K) or salicylic acid (SA) on proline content of wheat under stress and non stress conditions

Fig. 4: Effect of Kinetin (K) or salicylic acid (SA) on Total carbohydrates % of wheat under stress and non stress conditions

A complex essential role of carbohydrates in plant metabolism is well known as products of hydrolytic processes, energy production but also in a carbohydrate sensing and signaling systems. Carbohydrate may also function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor pressure.

Gluten content:

Gluten content scored significant differences across drought stress and K or SA treatments (Fig.5), whereas, gluten content was decreased under both stress treatment compared with control (no-stress). Application of K or SA generally increased significantly gluten content as compared with control (no-stress). Maximum gluten level was observed in plants grown under no-stress and applied with K or SA followed with plants grown under stress and applied with K or SA and surpassing control (no stress). Data indicates the positive effect of K or SA in protein synthesis and also its role in improving plant adaptation to drought stress as mentioned by Tasgin, [31]. Generally, drought stress poses numerous other adverse effects on plants, including decreased photosynthetic rate [32], stomatal closure, reduced transpiration and gas exchange, and structural damages or destructions that may lead to considerable reduction in total soluble protein content [29]. However, some of these adverse effects could be at least partially alleviated *via* plant growth regulators applications, as showed shown in our study and harmonious with various previous studies as Ashraf, *et al*. [13].

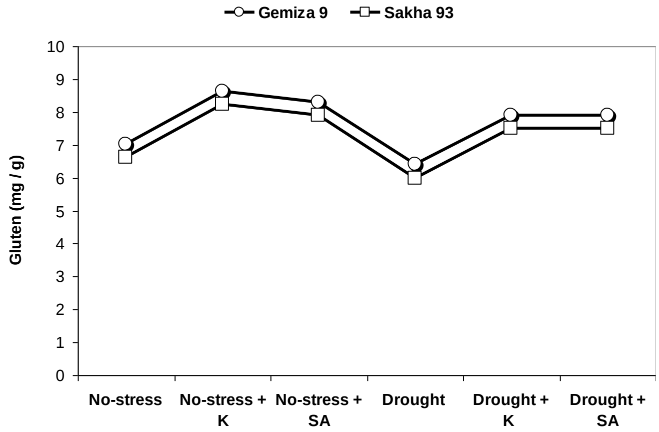
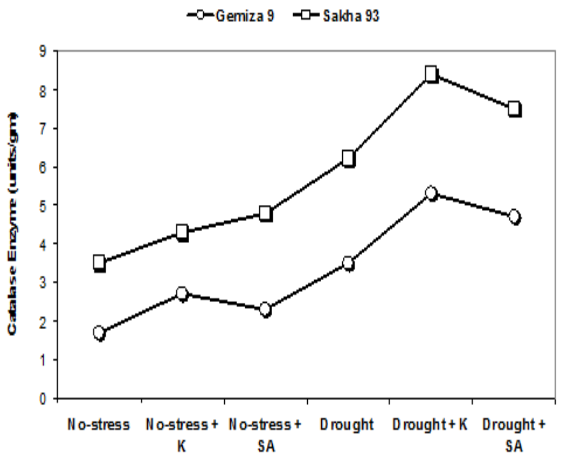


Fig. 5: Effect of Kinetin (K) or salicylic acid (SA) on gluten content of wheat under stress and non stress conditions

Antioxidant Enzyme:

Catalase activity was significantly affected by drought stress and K and SA treatments (Fig. 6). Application of K or SA resulted in a significant increase in CAT activity, the maximum increases in catalase activity was recorded at application with K or SA under drought stress. Kinetin application has more significant effect. The two cultivars showed similar trend. Also, a higher peroxidase activity was observed under drought stress than control (no-stress), and peroxidase activity (Fig. 7) increased in both cultivars under drought stress. Application of K or SA significantly increased the activity of POD. Generally, Catalase or peroxidase activity was increased under drought stress and the maximum increases in catalase and peroxidase activity was recorded at K or SA applications under drought stress. It worth mentioning, catalase and peroxidase activity was higher in Sakha 93 than Gemiza 9. During drought stress in wheat, activity of enzymatic antioxidant catalase increased to manage the oxidative stress [33, 34, 35, 36] which is synchronized with our results in present investigation. Thus, K or SA applications may result in enhancing plant drought tolerance by increasing the production of the various antioxidant enzymes. Drought induces oxidative stress in plants by generating of reactive oxygen species (ROS) [8] where the ROS such as O_2, H_2O_2 and OH radicals, can directly attack membrane lipids and increase lipid peroxidation [37]. Drought-induced overproduction of ROS increasing the content of malondialdehyde (MDA) .The content of MDA has been considered an indicator of oxidative damage [38].

To facilitate protection of cells against ROS under stress conditions, production of antioxidant enzymes can be stimulated *via* different means, including plant growth regulator applications [39]. There is a defensive system in plants, that is to say, plants have an internal protective enzyme-catalyzed clean up system, which is fine and elaborate enough to avoid injuries of active oxygen, thus guaranteeing normal cellular function [40]. The balance between ROS production and activities of antioxidative enzyme determines whether oxidative signaling and/or damage will occur [38]. To minimize the affections of oxidative stress, plants have evolved a complex enzymatic and non-enzymatic antioxidant system, such as low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS-scavenging enzymes (superoxide dismutase), peroxidase, catalase, ascorbate peroxidase [41].



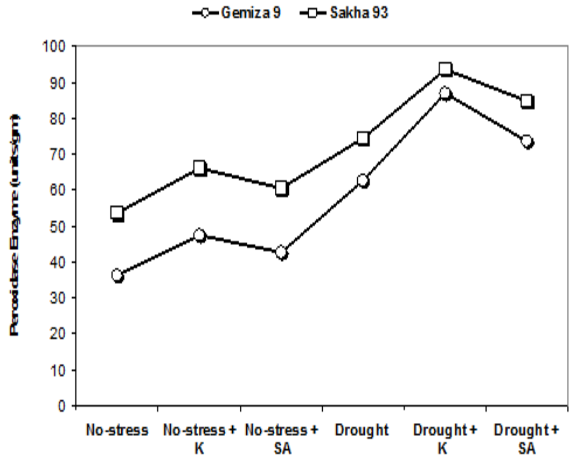
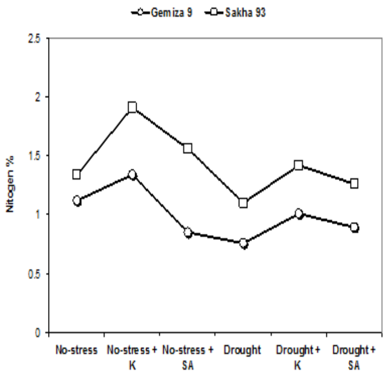


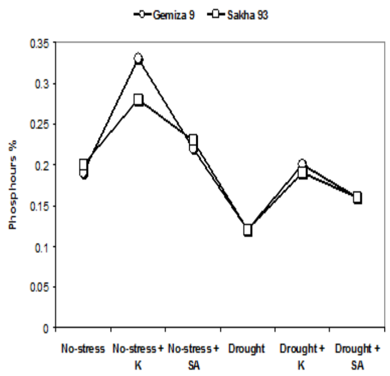
Fig. 6: Effect of Kinetin (K) or salicylic acid (SA) on catalase enzyme of wheat under stress and non stress conditions

Fig. 7: Effect of Kinetin (K) or salicylic acid (SA) on peroxidase enzyme of wheat under stress and non stress conditions

Macronutrient content:

Results in figures 8, 9, and 10 showed that macronutrient content (N, P, and K) in yielded grains of both cultivars was affected by drought stress compared with no stress conditions. In general, results that K or SA applications increased macronutrient content more than in drought application. Maximum macronutrient levels were recorded in plants grown under no stress and applied with K or SA. Reduction in ion metals concentration may be due to the dieback of the absorbing roots during the exposure of plants to drought conditions [42]. Moreover, Baque *et al*. [43] observed that mild and severe water stress significantly reduced the uptake of NPK in wheat plants compared to that of normal conditions. These results are in accordance with those obtained in wheat by Maria *et al*. [44].





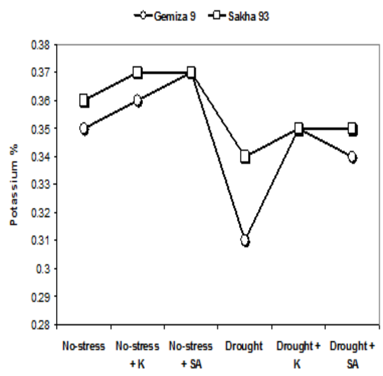


Fig. 8: Effect of Kinetin (K) or salicylic acid (SA) on nitrogen% of wheat under stress and non stress conditions

Fig. 9: Effect of Kinetin (K) or salicylic acid (SA) on Phosphorus% of wheat under stress and non stress conditions

Fig. 10: Effect of Kinetin (K) or salicylic acid (SA) on potassium % of wheat under stress and non stress conditions

Analysis of Variations in two cultivars affected by two different growth regulator treatments using ISSR markers:

Molecular markers analysis of eight DNA samples representing four treatments for both tested cultivars i.e. Gimeza 9 under no stress conditions (GNS), drought conditions treated with kienten (GDK), drought conditions treated with SA (GDSA), drought conditions (GD) and Sakha 69 under no stress conditions (SNS), drought conditions treated with kienten (SDK), drought conditions treated with SA (SDSA) drought conditions (SD) all were performed using three ISSR primer in order to explore the effect of the two different growth regulators treatments comparing with control on both tested cultivars (Figure 11). The ISSR reactions produced 35 scorable total bands, out of which 17 were found to be polymorphic.

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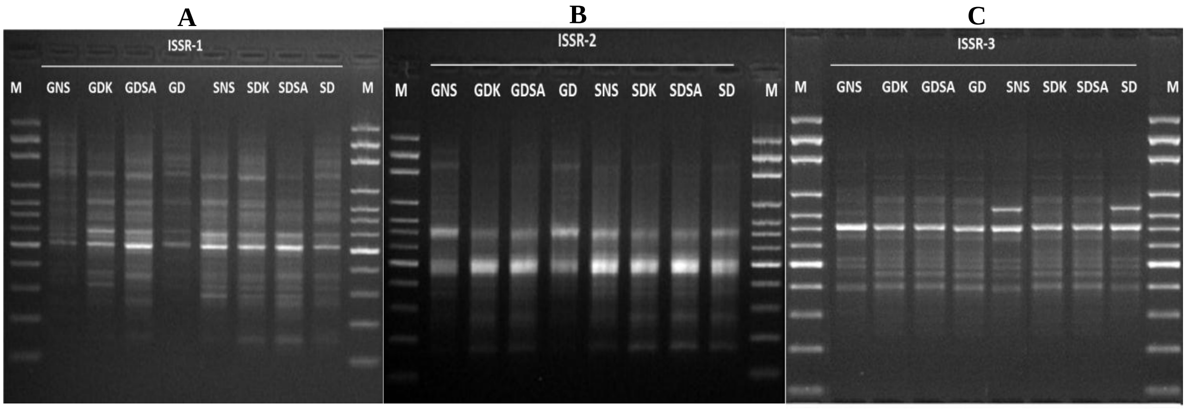

Fig.11: Agarose gel illustrates ISSR pattern variations of the two cultivars between control and two different growth regulators treatments as determined *via* spectrophotometry analysis.

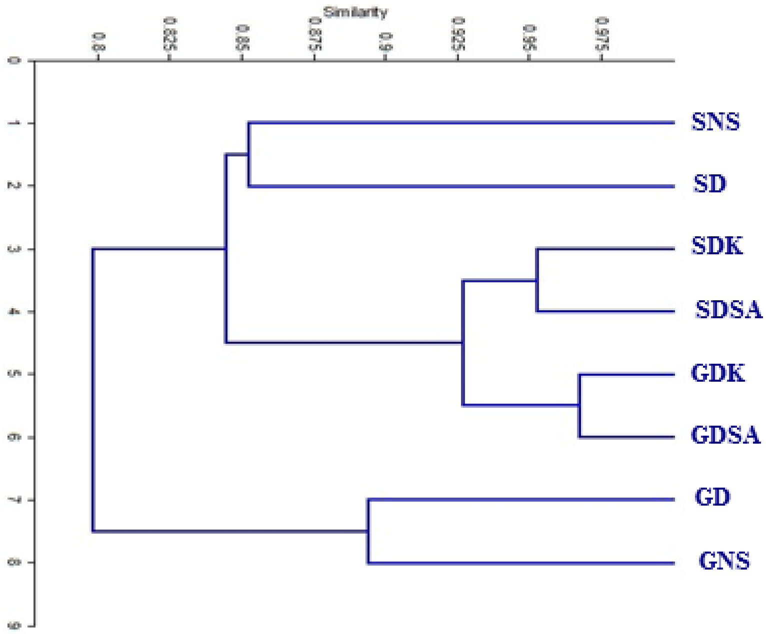

Fig. 12: Phylogenetic analysis based on combined data obtained from ISSR markers.

Drought stress is a major risk for international food security. Morphological and physiological responses to drought are controlled by various polygenes which regulate drought resistance [45]. Numerous physiological and biochemical limitations and adverse effects were imposed by drought across plant species and genotypes [2, 46]. Wheat productivity is commonly affected by of drought. Plant growth and productivity are adversely affected by water stress [4]. ISSR markers are simple, PCR based, locus specific, more reliable, reproducible and typically co-dominant markers.

A dendrogram based on UPGMA analysis of the amplicons obtained from ISSR markers was constructed (Figure 12). The dendrogram comprise two main clusters, the first main cluster (the major) was subsequently divided into two subclusters; the first main subcluster comprised into two sub-subclusters. The first sub-subcluster including the SNS and SD treatments. Meanwhile, the second sub-subcluster divided into two sub-sub-subcluster. the first sub-sub-subcluster including the SDK and SDSA treatments. Meanwhile, the second sub-sub-subcluster including the GDK and GDSA treatments. Meanwhile, the second main subcluster including the GD and GNS treatments. The obtained results from ISSR marker systems successfully revealed a discriminative pattern between the DNA isolated from seeds of control and treatments. The cluster analysis results exhibited that the two cultivars cv. Sakha 93 and Gemiza 9 under either stress or under no stress were clustered into a main cluster and this may be due to the genetic differences between both cultivars. On the other hand, Sakha cultivar with all treatments and also Giza cultivar under stress treated with either SA or K were clustered into the other main cluster. It was revealed that the similarity percentage of Sakha 93 cultivar treatments under no stress and under drought stress similarity percentage scored 85%. In contrast, Sakha cultivar treatment under no stress and treatments either by K or SA under drought stress scored 81 and 86%; respectively. Moreover, it was demonstrated that Gemiza 9 cultivar treatments similarity between under no stress and under drought stress was very high and scored 89%. On the other hand, Gemiza cultivar treatment under no stress and treatments either treated by K or SA under drought stress scored 81 and 78%; respectively. Examination of ISSR-PCR assay mirrored minor variations in clustering pattern. The explication could furthermore be forwarded that the two cultivars are of common gene pool. In parallel, all the treatments appropriate for drought conditions were clustered in a distinct main cluster. Likewise, treatments to tolerate drought of both Sakha 93 and Gemiza 9 were also clustered together. It might be suggested that determinants here in our study were corresponding to the gene complexes responsible for drought and adaptation reactions. In this perspective, Singh *et al.* [47] studied some ecological adverse effects by ISSR analysis where results indicated that ISSR had the capability to differentiate and track these variations. Such variations are taken as a basis of molecular characterization of the cultivars and genotypes [48, 49]. However, influence of adaptation on classification of cultivars remained distinct. Spontaneously, varied minor genetic changes which have occurred in a particular drought treatments seem to be an adaptation which serves to discriminate between the two tested cultivars. Also, adaptation is ruled by several gene complexes. Moreover, adaptation is a function of the genes controlled by range of biochemical and physiological processes regulations during growth and development and how well these genes are harmonized with the existing environment. Furthermore, adaptation is a result of genetic mechanism which stimuluses the matching of growth and development processes within environment [50]. In brief, process of adaptation is viewed as changes in the cultivars genetic structure as they accumulate genes or a change in gene frequencies which to be better matching with growth and development within environment [51]. Therefore, wheat drought tolerance is a result of number of component traits effects. Though, it could not be detected by ISSR markers that these determinants are genetic evolution and it is suggested that it requires further evaluation by genome sequence.

Therefore, genes responsible for some important components for drought adaptation seem to be represented clearly by ISSR. Consequently, it could be concluded that ISSR were more robust and could detect even minor variations more efficiently and correspondingly fast and reliable for genetic characterization for the two tested cultivars of wheat treated to adopt drought stress [52, 53, 54].

Conclusion:

Drought is a worldwide problem, constraining global crop production and quality seriously, and recent global climate change has made this situation more serious. Drought stress affects the growth, dry mater and harvestable yield in plants. The growth regulators treatments (kinetin and salicylic acid) reduced the damaging action of water deficit on wheat growth and accelerated a restoration of growth processes. Applications caused increases in yield under normal or drought stress conditions; however, foliar application of kinetin caused maximum increase in grain yield of wheat under control or drought stress conditions. Also, using ISSR was robust and it detects minor variations fast, efficiently and reliable for the two tested wheat cultivars treated to adopt drought stress.

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