Universidade de Trás-os-Montes e Alto Douro

Effectiveness of Ascorbic Acid and Zinc as drought antagonists in bread wheat plants monitored by different DNA markers

Dissertação de Mestrado em Genética Molecular Comparativa e Tecnológica

João Tiago Roque Alves

Orientador: Professor Doutor José Eduardo Lima-Brito

Co-Orientador: Doutora Ana Isabel Ferreira de Carvalho

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Eficiência do Ácido Ascórbico e Zinco como antagonistas da seca em plantas de trigo mole monitorizada por diferentes marcadores de DNA

RESUMO

O trigo mole é um cereal economicamente importante a nível mundial e devido às alterações climáticas, a seca é frequente afetando o rendimento e a qualidade desta cultura. O Ácido Ascórbico (AsA) e o Zinco (Zn) podem atuar como antagonistas da seca minimizando os efeitos negativos deste stresse abiótico.

A seca e outros stresses abióticos geram instabilidade genómica nas plantas que pode ser monitorizada por marcadores moleculares. "Conserved DNA Domain Polymorphism" (CDDP) e "Start Codon Targeted" (SCoT) são "gene-targeted" markers capazes de detetar variação em regiões genómicas funcionais; marcadores baseados em retrotransposões (RTNs) podem monitorizar a atividade dos RTNs e os marcadores "Random Amplified Polymorphic DNA" (RAPD) podem detetar instabilidade genómica ao longo do genoma.

Neste trabalho, pretendeu-se testar a eficiência de "priming" de sementes e/ou a aplicação foliar com AsA ou sulfato de Zn heptahidratado ($ZnSO_4.7H_2O$) para mitigação da seca em plantas de trigo mole da cv. Jordão através da avaliação da instabilidade genómica em plantas tratadas relativamente às plantas controlo ambas em dois regimes hídricos (rega e seca) usando marcadores CDDP, SCoT, "Inter-Retrotransposon Amplified Polymorphism" (IRAP), "Retrotransposon-Microsatellite Amplified Polymorphism" (REMAP), "inter-Priming Binding Site" (iPBS), "Inter-Simple Sequence Repeat" (ISSR) e RAPDs.

A instabilidade genómica foi refletida pelo aparecimento de novas bandas e pela perda de bandas nas plantas tratadas relativamente às plantas controlo. Assim, com base nas percentagens de polimorfismo obtidas com os diferentes marcadores de DNA em todos os tratamentos e em ambos os regimes hídricos, extrapolou-se o antagonista da seca mais adequado ao trigo mole.

Os polimorfismos gerados poderão ter sido devidos a alterações na sequência do DNA quer dos locais de hibridação dos primers quer na região genómica entre eles.

Ambos os marcadores "gene-targeted", SCoT e CDDP permitiram uma análise da variação do DNA ao longo do genoma e foram adequados à deteção de instabilidade genómica em plantas de trigo mole em stresse abiótico. Contudo, consideraram-se os

resultados CDDP mais fiáveis uma vez que foram produzidos com base em primers especifícos para genes de resposta ao stresse hídrico previamente isolados em trigo mole. As técnicas IRAP e REMAP evidenciaram as taxas de polimorfismo mais elevadas, corroborando a elevada actividade insercional e transcripcional dos RTNs em plantas sob stresse abiótico como a seca, a qual pode ser detetada por marcadores baseados em RTNs. Os marcadores RAPD demonstraram ser úteis para uma análise genómica relatiavmente à deteção de instabilidade genómica em plantas de trigo mole sob stresse abiótico.

Em suma, integrando todos os dados moleculares e estatísticos obtidos neste trabalho, o "priming" de sementes com Zn (Zn priming) parece ser o tratamento mais adequado para plantas de trigo mole sob seca.

Palavras-chave: Antagonistas da seca; marcadores moleculares; remodelação genómica; stresse abiótico; *Triticum aestivum* L. em. Thell..

Effectiveness of Ascorbic Acid and Zinc as drought antagonists in bread wheat plants monitored by different DNA markers

ABSTRACT

Bread wheat is economical important worldwide and due to the climate changes drought is frequent and it is affecting the yield and quality of this crop. Ascorbic Acid (AsA) and Zinc (Zn) can act as drought antagonists minimizing the negative effects of this abiotic stress.

Drought and other abiotic stresses generate genomic instability in plants that can be monitored by molecular markers. Conserved DNA Domain Polymorphism (CDDP) and Start Codon Targeted (SCoT) are gene-targeted markers that are able to assess variation in functional genomic regions; retrotransposons (RTNs)-based markers may moniter the RTNs activity and Random Amplified Polymorphic DNA (RAPD) are able to screen genomic instabilities throughout the genome.

In this study, we intend to test the effectiveness of seed priming and/or foliar application with Ascorbic Acid (AsA) or Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) for drought mitigation in bread wheat plants of cv. 'Jordão' by evaluating the genomic instability in treated plants relative to control plants both under two water regimes (watering and drought) using CDDPs, SCoTs, Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP), inter-Priming Binding Site (iPBS), Inter-Simple Sequence Repeats (ISSRs) and RAPDs.

The genomic instability was reflected by the appearance of new bands and loss of bands in treated plants relative to control. Therefore, based on the percentage of these polymorphic bands achieved with the different DNA markers in all treatments of both water regimes, it was extrapolated the most suited drought antagonist for bread wheat.

The polymorphisms were generated due to alterations in the DNA sequence that may affect both priming binding sites and the genomic region between them.

Both SCoT and CDDP gene-targeted markers allowed a genome-wide DNA variation analysis being suitable for detection of genomic instability in bread wheat plants under abiotic stress. However, we considered more reliable the CDDP results since their production was based on primers specific to water stress responsive genes previously isolated in bread wheat.

The IRAP and REMAP techniques evidenced higher percentages of polymorphism, corroborating the assumption that plants under abiotic stress such as drought have higher RTNs insertional and transcriptional activities that could be detectable by RTNs-based markers. RAPD assays demonstrated to be a useful tool in order to do a genome wide analysis for the detection of genomic instabilities in bread wheat plants under abiotic stress.

Overall, integrating all the molecular and statistical results achieved in this work, seed priming with Zn (Zn priming) appeared to be the most suitable treatment for bread wheat plants under drought.

Keywords: Abiotic stress; bread wheat; drought antagonists; genomic remodelling; molecular makers; *Triticum aestivum* L. em. Thell..

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List of abbreviations

$(NH_4)_2SO_4$ – Ammonium sulfate
$[Ca(NO_3)_2]$ – Calcium nitrate
µL - Microliters
µM - Micromolar
ABA - Abscisic acid
ANOVA – Analysis of variance
APX – Ascorbate peroxidase
AsA – Ascorbic Acid
B - Boron
BaP - Benzo[*a*]pyrene
BARE-1– Barley retroelement 1
bp – Base pair
Br - Bromide
Cd – Cadmium
CD - Control plants under drought
CDDP - Conserved DNA Domain Polymorphism
cDNA – Complementary DNA
Cr – Cromium
cv. - Cultivar
CTAB – Cetyl trimethylammonium bromide
CW – Control plants in watering regime
DNA – Desoxirribonucleic acid
dNTP - Deoxynucleotide
Hg - Mercury
INIAV – Instituto Nacional de Investigação Agrária e Veterinária
iPBS - inter-Priming Binding Site
IRAP - Inter-Retrotransposon Amplified Polymorphism
ISSR - Inter-Simple Sequence Repeat
K – Potassium
KCl – Potassium chloride
KH_2PO_4 – Potassium dihydrogen phosphate
KOH – Potassium hydroxide
LARD - Large RTNs derivatives
LINE - Long Interspersed Repetitive Elements
LTR - Long Terminal Repeat
$MgCl_2$ – Magnesium chloride
MITE - Miniature Inverted-Repeats Transposable Elements
mM - Millimolar
ng - nanogram
ºC – Celsius degrees
P – Phosphorus
PBS – Priming binding site
PCoA - Principal Coordinates Analysis
PCR – Polymerase Chain Reaction
PIMP – Putative Integral Membrane Protein
PLSD - Protected Least Significant Difference (Post hoc Fisher's test)

PNP - Paranitrophenol
RAPD - Random Amplified Polymorphic DNA
REMAP - Retrotransposon-Microsatellite Amplified Polymorphism
RNA – Ribonucleic acid
ROS – Reactive oxigen species
RTN - Retrotransposon
SCoT – Start Codon Targeted
SE - Standard error
SINE - Short Interspersed Repetitive Elements
SSR - Simple Sequence Repeat
TBE – Tris-Borate-EDTA
TE – Transposable element
TRIM - Terminal-Repeat In Miniature (RTN)
U - Units
UTAD – Universidade de Trás-os-Montes e Alto Douro
Zn - Zinc
$ZnSO_47H_2O$ – Zinc sulfate heptahydrate

1. GENERAL BIBLIOGRAPHIC REVIEW

1.1. Introduction

Wheat is the second most important crop produced worldwide representing 60% of the daily calories consumed in several developing countries having a major impact on human health (Karim *et al*. 2012; Ahmed *et al*. 2016).

Due to the global climate changes, severe and frequent drought episodes occur in several regions of the world particularly in the Mediterranean basin, which affects the production and quality of the wheat grain (Karim *et al*. 2012; Shiferaw *et al*. 2013; Wang *et al*. 2014; Steinemann *et al*. 2015; Ahmed *et al.* 2016; Hafez and Gharib 2016; Nuttall *et al*. 2017).The wheat grain yield decreases under reduced water availability and when this stress is combined with others (Shiferaw *et al*. 2013).

Wheat production in World reached 759.590 millions of tons in 2016/2017, and the forecast production for 2017/2018 is 750.120 millions of tons [1]. In fact, the international prices of wheat, including those achieved in Europe and Mediterranean region, increased during the last summer and were around 28% higher relative to the price reached in the summer of 2016 [2]. This increase of wheat price was due to reduced availability of high-quality grain as consequence of the dry and hot weather (FAO 2017).

As plants respond to water stress by decreasing the transpiration rate, the rate of carbon sequestration and growth also decrease. Indeed, water deficits can lead to tissue dehydration, decrease of leaf chlorophyll and damage of the photosynthetic apparatus, alterations of metabolic processes such as nutrient uptake and flowers sterility during the reproductive growth (Barlow *et al*. 2013; Shiferaw *et al*. 2013; Hafez and Gharib 2016; Nuttall *et al*. 2017). Water stress as well as other abiotic stresses are associated with nutrient imbalance and production of reactive oxygen species (ROS) that consequently reduce the growth and yield of the crops (Batool *et al*. 2012).

Efforts have been made in order to develop short-term strategies that mitigate the negative consequences of drought in crops, but also long-term approaches such as the selection of genotypes more tolerant to abiotic stress. Regarding the short-term strategies, it has been reported the effectiveness of Ascorbic Acid (AsA) and Zinc (Zn) in the mitigation of the negative consequences of abiotic stress in different crops, including wheat.

Several studies reported that drought and other abiotic stresses induce genetically and epigenetically programmed responses that cause genomic instability which can be evidenced as polymorphism in plants under stress (Liu *et al*. 2005, 2007; Atienzar and Jha 2006; Cencki *et al*. 2009; Kekec *et al*. 2010; Fan *et al*. 2014)

Molecular DNA markers are valuable tools for detecting genetic polymorphisms as result of stress (Liu *et al*. 2005, 2007; Atienzar and Jha 2006; Cencki *et al*. 2009; Kekec *et al*. 2010; Fan *et al*. 2014).

1.2. **Triticum aestivum *L.: origin and economic importance***

Between eight and ten thousand years ago, the first permanent agricultural settlements of the Fertile Crescent developed bread wheat from emmer and einkorn grasses (Shiferaw *et al*. 2013).

Taxonomically, the bread wheat (*Triticum aestivum* L. em. Thell.), is included in the kingdom Plantae, subkingdom Viridiplantae, infrakingdom Streptophyta, superdivision Embyophyta, division Tracheophyta, subdivision Spermatophytina, class Magnoliopsida, superorder Lilianae, order Poales, family Poaceae, tribe Triticeae, subtribe Triticinae and genus *Triticum* (Breiman and Graur 1995; [3]).

The tribe Triticeae is evolutionarily recent and encloses members from different genera and with various ploidy levels being able to hybridize among them (Breiman and Graur 1995; Lima-Brito *et al*. 1998, 2006; Carvalho *et al*. 2008, among many others). In 1918, Sakamura established the basic chromosome number of the genus *Triticum* L. as x = 7. Hence, this genus includes diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) and hexaploid (2n = 6x = 42) species (Kihara 1944).

The modern bread and durum wheat are polyploids that arose from spontaneous hybridization events which combined individual homoeologous diploid genomes originated from a common ancestor (Sabot *et al.* 2005; Fig. 1). Durum wheat, *Triticum turgidum* ssp. *dicoccoides* (2n = 4x = 28, AABB genome) resulted from the hybridization of *Triticum urartu* (A^uA^u genome) with a still unidentified species of the section *Sitopsis,* probably closely related to the actual *Aegilops speltoides* (SS genome; Levy and Feldman 2002), that is considered the donor species of the B-wheat genome. A species derived from this tetraploid wheat (AABB), then hybridized with *Aegilops tauschii* (DD genome), resulting after

domestication, in the modern bread wheat (*T. aestivum*, 2n = 6x = 42, AABBDD) (Huang *et al.* 2002; Salamini *et al.* 2002).

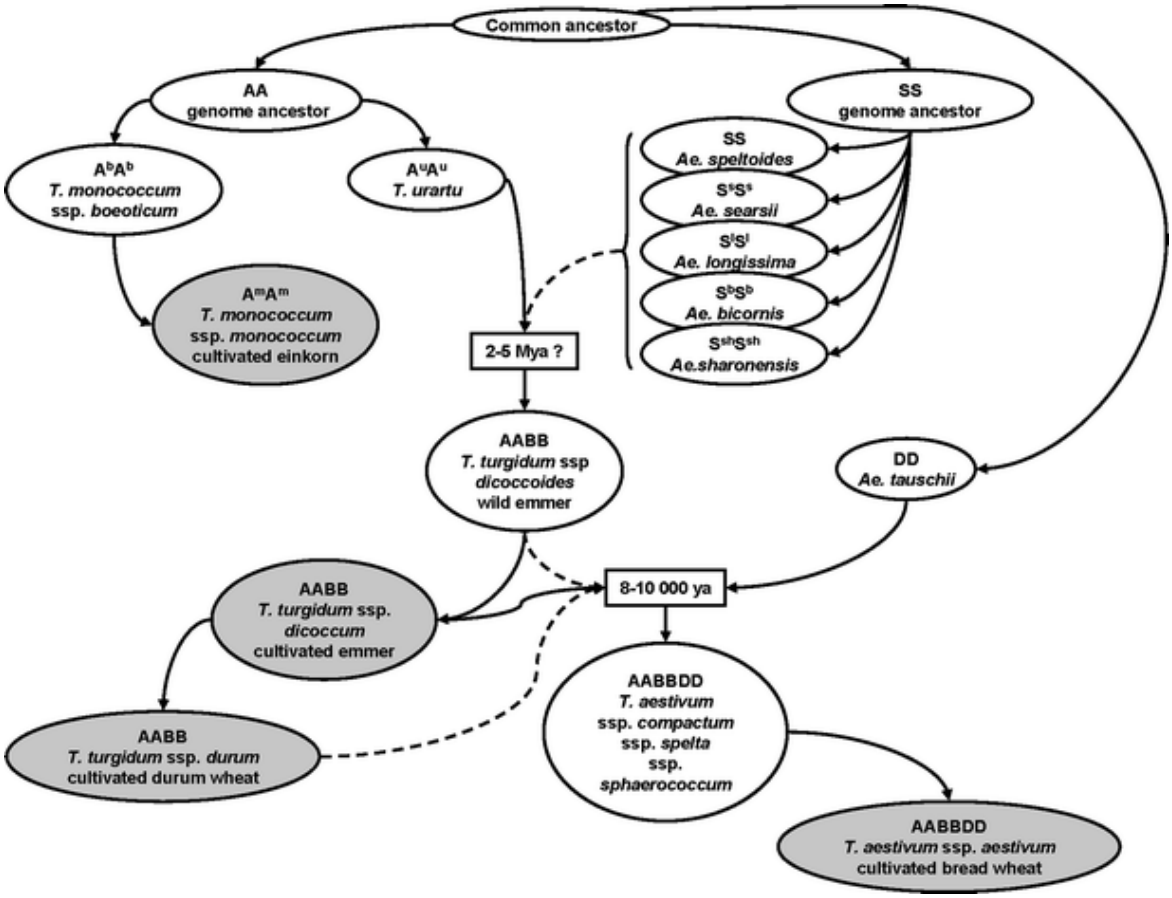


Figure 1. Evolutionary history of polyploid wheats. The shaded circles represent the cultivated forms of wheat. The broken lines represent the other possible origins of the AB genome during formation of the hexaploid.Source: Sabot et al (2005).

Due to the high economical and nutritional importance of wheat, it has been used as model species in several studies focusing strategies for abiotic stress mitigation (Karim *et al.* 2012; Shiferaw *et al*. 2013; Ahmed *et al.* 2016).

The total global demand for wheat quadrupled since the 1960s (Shiferaw *et al*. 2013). Wheat is the most important source of protein and provides on average about 21% of the daily dietary protein intake and around 50% if we look to the contributions of total cereals consumption (Shiferaw *et al*. 2013).

1.3. Short term strategies for abiotic stress mitigation

Many methodologies have been used in order to mitigate the effects of abiotic stresses, such as: the conventional breeding (selection and hybridization), modern methods (mutation breeding, polyploidy breeding, genetic engineering) (Jisha *et al*. 2013) and agronomic biofortification (Cakmak *et al*. 2010; Bouis *et al*. 2011; Nawaz *et al*. 2013).

The conventional breeding techniques have several limitations, like the need of huge manpower and energy. Attempts were made in order to produce plant varieties with improved drought and salinity tolerance but these attempts failed due to complexity and multigenic nature of drought and salinity tolerance traits (Cushman and Bohnert 2000; Flowers *et al*. 2000).

The modern methods are also limited. Despite the fact that targeted introduction of individual traits into elite crop lines can be reasonably fast and predictable, this is not a simple process not only by the effects of gene silencing and pleiotropy but also because they are expensive and have several biosafety regulations and restrictions (Flowers *et al*. 1997; Gust *et al*. 2010; Jisha *et al*. 2013).

In order to overcome those limitations, it has become imperative to find alternative effective solutions to mitigate the impacts of abiotic stresses that should be simple, affordable and easily adopted by farmers (Jisha *et al*. 2013).

Agronomic biofortification is the process of increasing the micronutrient density of staple crops, thus improving the nutritional content and providing an inexpensive, cost-effective and sustainable mean of delivering micronutrients (Cakmak *et al*. 2010; Bouis *et al*. 2011). This enables the reduction of the number of severely malnourished people, especially in poor areas of the Globe that have a limited access to commercially marketed fortified foods and supplements (Bouis *et al*. 2011). The priming of seeds was identified as way of improving the micronutrient content of crops and so it is a valid method to achieve the biofortification of crops (Harris *et al*. 2008; Nawaz *et al*. 2013).

1.3.1. Seed priming

Seed priming is a water-based technique that allows a controlled pre-treatment of the seeds to trigger the metabolic processes that are usually activated during the early phase of germination (Paparella *et al.* 2015), and has been reported as an effective method of improving stress tolerance in plants (Jisha *et al*. 2013, Jisha and Puthur 2016).

There are several types of seed priming (immersion of seeds): (i) in sterilized water kept at appropriate temperature - hydropriming; (ii) in solutions cointainning the limitant nutrient instead of pure water - nutrient priming; (iii) in osmotic solutions at low water potential, facilitating the control of water uptake - osmopriming; (iv) in solutions containing conventional disinfectants, natural substances and agrochemicals to prevent microbial contaminations - chemical priming; (v) in solutions containing plant growth regulators and hormones - hormonal priming; (vi) in solutions containing bacterial agents to improve the establishment of the crop - biological priming; (vii) in a solution design to maintain the correct redox state of cells - redox priming; and (viii) by mixing seeds with solid material able to properly adjust the moisture content, allowing to control water uptake - solid matrix priming (Jisha *et al*. 2013; Farooq *et al*. 2014; Sharma *et al*. 2014; Paparella *et al.* 2015; Lutts *et al.* 2016).

The benefits of seed priming are widely described, including the synchronous and fast germination in a wide range of temperatures, reduced photo- and thermo-dormancy, improvement of competition with weeds and pathogens and growth (Jisha *et al*. 2013 Paparella *et al.* 2015; Jisha and Puthur 2016).

The mineral or nutrient status of plants influences the ability of plants to resist to abiotic stresses. Therefore, nutrient priming improves the nutritional value of the crop, yield and/or the resistance to abiotic stress (Jisha *et al*. 2013). Harris *et al*. (2008) demonstrated that seed priming with Zn solutions improved the grain yield in chickpea and wheat. Yagmur and Kaydan (2008) performed seed priming with Potassium dihydrogen phosphate (KH_2PO_4) solutions and verified that this approach was effective in the alleviation of salt stress in triticale. Seed priming with salicylic acid (SA) improved the cold tolerance in maize (Farooq *et al*. 2008) and mitigated the effects of salt stress in *Vicia faba* L. (Azooz 2009). Guan *et al*. (2009) demonstrated that seed priming with chitosan was successful to mitigate the effects of low temperatures.

Seed priming can also be performed with antioxidants such as AsA. The pretreatment of seeds with AsA enhanced the germination properties of *Agropyron elongatum* under salt stress (Tavilli *et al*. 2009) and improved the tolerance to cold and salinity in *Capsicum annuum* L. (Yadav *et al*. 2011).

1.3.2. Foliar application

The availability of macro- and micronutrients to plants is influenced by the physical and chemical properties of the soil. When the availability of nutrients does not meet the crop needs, it induces abiotic stress (Arif *et al*. 2006; Zoz *et al*. 2012). Most of the micronutrients are readily fixed in alkaline soils and become unable to be absorbed by the plant roots or incapable to be transported (Arif *et al*. 2006; Zoz *et al*. 2012).

The foliar spraying with solutions based on micronutrients, antioxidants or other agents that mitigate the abiotic stress effects constitutes another alternative approach of exogenous supply of plant requirements, namely, under environmental constraints, in order to ensure the proper plant growth, development, and metabolism (Arif *et al*. 2006).

Previous studies demonstrated that the application of micronutrients by foliar spraying is equally or more effective than their application to soil, overcoming the nutrient deficiencies in the soil (Grewal *et al*. 1997; Modaihsh 1997; Torum *et al*. 2001; Arif *et al*. 2006).

Kaya *et al*. (2001) demonstrated that the foliar application of Potassium (K) and Phosphorus (P) was able to mitigate the effects of salt stress in *Lycopersicon esculentum*. Murillo-Amador *et al*. (2006) performed foliar application of Calcium nitrate [$Ca(NO_3)_2$] and mitigated the damage caused by salt stress in *Vigna unguiculata* L.. Akram *et al*. (2007) showed that the foliar application of Potassium hydroxide (KOH) was able to mitigate the effects of salt stress in *Helianthus annuus* L.. Dolatabadian *et al.* (2008) concluded that the foliar spraying of AsA reduced the salt stress in *Brassica napus* L.. Movahhedy-Dehnavy *et al*. (2009) showed that the foliar application of Zn and Manganese (Mn) successfully reduced the effects of water stress in *Carthamus tinctorius* L.. Zoz *et al*. (2012) tested the foliar application of Zn in wheat and reported the increase in both number of fertile tillers and yield.

Since AsA and Zn were already described in the literature as having a positive effect on crops under stress conditions, it is important to understand their effectiveness in the mitigation of drought effects in bread wheat whose production has been negatively affected

by this abiotic stress. Besides, the exogenous supply of an antioxidant (AsA) and an essential micronutrient (Zn) to wheat would improve its nutritional value.

1.3.3. Ascorbic Acid (AsA)

There are three main biological functions defined for AsA, a unique low-molecular-weight-antioxidant: enzyme cofactor, radical scavenger and donor/acceptor in electron transport either in plasma membrane and chloroplasts (Davey *et al*. 2000; Smirnoff and Wheeler 2000; Hafez and Gharib 2016).

According to Davey *et al*. (2000), AsA it is involved in: (i) the synthesis of hydroxyproline-rich proteins; (ii) the cell expansion and division; (iii) the activation of myrosinase - an enzyme that catalyses the hydrolysis of glucosinolates of D-glucose and an aglycone fragment; (iv) the biosynthesis of some plant hormones, such as ethylene and gibberellic acid; and (v) in terminating radical chain reactions, acting as a donor/acceptor of electrons and serving as substrate for enzymes that scavenge ROS, such as ascorbate peroxidase (APX).

Several studies pointed out AsA as an antagonist agent of abiotic stress (Shalata and Neumann 2001; Athar *et al*. 2008; Beltagi 2008; Hafez and Gharib 2016).

AsA treatments through seed priming, foliar application or direct application on the soil, stimulate the accumulation and synthesis of phytohormones and total soluble carbohydrates that alleviate the abiotic stress constraints; improve yield; protect the photosynthetic apparatus from the oxidative damage; and increase the enzymatic activity of catalase and peroxidase that eliminate ROS produced during stress (Farooq *et al*. 2012; Sadak 2015; Hafez and Gharib 2016).

In the particular case of wheat, the exogenous application of AsA *via* seed priming or direct application on the soil proved to be very effective in the mitigation of the adverse effects of water stress, probably, due to the activation of catalase and peroxidase (Hafez and Gharib 2016). Besides, the direct application of AsA on the soil stimulated the yield-related traits, mainly in the watered plants, and also demonstrated to be suitable for the increase of wheat productivity under water stress (Hafez and Gharib 2016).

1.3.4. Zinc (Zn)

Zinc (Zn) is an essential cofactor required for the structure and function of numerous proteins and an important component of enzymes for protein synthesis, energy production and maintenance of the structural integrity of the biological membranes. Most of the Zn enzymes are involved in regulation DNA-transcription, RNA-processing and translation. Besides, several transcription factors and enzymes involved in DNA or RNA synthesis or maintenance are Zn-dependent (Hänsch and Mendel 2009).

Zn deficiency is common in alkaline soils and affects widely the cereals cultivation (Grotz and Guerinot 2006; Zoz *et al*. 2012). Besides, the Zn uptake is even more reduced when plants are under water stress (Karim *et al.* 2012). However, the exogenous application of Zn in proper amounts can increase both the drought resistance and yield (Karim *et al.* 2012; Zoz *et al*. 2012), improve the seeds development and overcome the delay in maturity observed in Zn-deficient plants (Hänsch and Mendel 2009).

Different authors reported that in Zn-treated plants, the effects of abiotic stress including drought, were alleviated (Zhao *et al*. 2005; Bagci *et al.* 2007; Karim *et al*. 2012; Zoz *et al*. 2012). Besides, the reviewed literature evidenced that the exogenous application of antioxidants such as AsA or micronutrients like Zn, through seed priming before sowing or foliar spraying before or at the reproductive stage, can mitigate the effects of abiotic stresses, improve the germination rate, seedlings growth and yield (Khan *et al*. 2006; Athar *et al*. 2008; Bagci *et al*. 2007; Hassanein *et al*. 2009; Farooq *et al*. 2012; Karim *et al*. 2012; Malik and Ashraf 2012; Sharma *et al*. 2014; Sadak 2015; Zoz *et al*. 2012; Ahmed *et al*. 2016; Hafez and Gharib 2016).

1.4. Genomic consequences of drought

Drought and other abiotic stresses lead to various genetic and epigenetic responses (Kim *et al*. 2015; Liu *et al*. 2015; Pandey *et al*. 2016). The consequent instability or remodelling of the genome can be evidenced as polymorphism in plants under stress (Madlung and Comai 2004; Liu *et al*. 2005, 2007; Atienzar and Jha 2006; Cencki *et al*. 2009; Chinnusamy and Zhu 2009; Kekec *et al*. 2010; Boyko and Kovalchuk 2011; Luo *et al*. 2012; Alzohairy *et al*. 2014).

Plant genomes respond to abiotic and biotic stresses through transcriptional and transpositional activation of transposons, structural and sequence changes, alterations in

transcription and regulation of affected genes (Grandbastien 1998; Madlung and Comai 2004; Huang *et al*. 2012; Alzohairy *et al*. 2014; Fan *et al*. 2014; Makarevitch *et al*. 2015; Negi *et al*. 2016). The nested organization of transposable elements (TEs) and genes contributes for their roles in gene expression regulation in response to environmental stimuli, in the induction of phenotypic plasticity and adaptation to stress, and are also the main determinants of polymorphism (Fan *et al*. 2014; Makarevitch *et al*. 2015; Negi *et al*. 2016).

DNA markers are able to screen genetic polymorphisms throughout the whole genome that may arise from DNA damage or mutational events like point mutations, small insertions and deletions in response to stress (Liu *et al*. 2005; Atienzar and Jha 2006; Fan *et al*. 2014).

Random Amplified Polymorphic DNA (RAPD) and/or Inter-Simple Sequence Repeat (ISSR) markers have been successfully employed for the assessment of genomic instability in plants regenerated *via* organogenesis (Guo *et al*. 2006); in genotoxicity studies related to heavy metals exposure in barley (Liu *et al*. 2005), rice (Liu *et al*. 2007), bread wheat and bean (Kekec *et al*. 2010). RAPDs and ISSRs have also been used for molecular characterization and identification of specific markers in drought tolerant and susceptible bread wheat cultivars (Deshmukh *et al*. 2012).

Based on the reviewed literature, the combination of CDDP, SCoT, IRAP, REMAP, ISSR, iPBS and RAPD used in the present work have never been attempted before for the assessment of genomic instability in bread wheat plants under drought.

Although the atual and wide availability of DNA markers, in the following items only those used in this work will be presented.

1.5. Gene-targeted markers

Molecular markers from the transcribed portion of the genome have potential to be used in plant genotyping because they are able to reveal polymorphisms that could be directly related to gene function (Poczai *et al*. 2013).

The dominant Start Codon Targeted (SCoT) (Collard and Mackill 2009a) and Conserved DNA Domain Polymorphism (CDDP) (Collard and Mackill 2009b) are gene-targeted markers (see Poczai *et al*. 2013) that use a single primer per reaction. These DNA markers are cost-effective, versatile, reproducible, and produce fast results (Collard and Mackill 2009a,b; Poczai *et al*. 2013).

1.5.1. SCoT markers

The SCoT technique is based on the fact that the ATG start codon is flanked by short conserved regions of the plant genes on both DNA strands (Poczai *et al*. 2013) to which SCoT primers were designed (Collard and Mackill 2009a). The amplicons generated are distributed within gene regions that contain genes on both plus and minus DNA strands (Poczai *et al*. 2013) (Fig. 2).

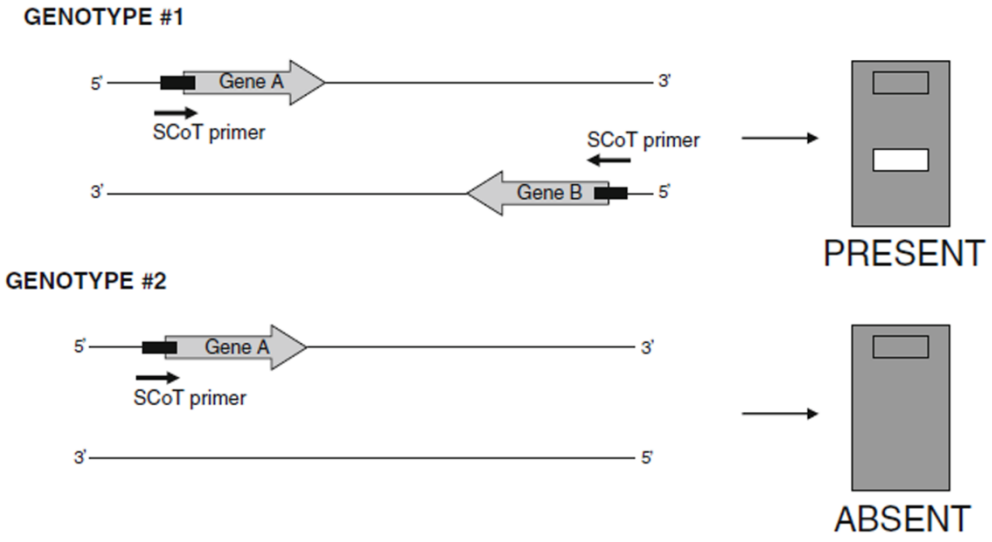


Figure 2. Diagram showing the amplification principle of SCoTs. Source: Collard and Mackill (2009a).

SCoTs are reproducible markers due to the primer length and high annealing temperature (50 ºC) (Collard and Mackill 2009a; Poczai *et al*. 2013) being useful for the further target of genes (Bhawna *et al*. 2017). SCoTs have been used alone or in combination with other DNA markers, such as CDDP, for assessment of genetic diversity, populations structure, gene flow, and DNA fingerprinting (Gorji *et al*. 2011; Bhattacharyya *et al*. 2013; Cabo *et al*. 2014a; Huang *et al*. 2014; Atia *et a*l. 2017; Bhawna *et al*. 2017). Recently, the technique cDNA-SCoT was used as complementary tool in one study of gene expression to profile tolerant and sensible plants to salt-stress (Al-Qurainy *et al*. 2017).

1.5.2. CDDP markers

Sequences of gene families present in multiple copies in the plant genome can be amplified by short primers. Across functional domains of well-studied plant genes, these short tags can be used to achieve informative banding patterns that can be used in several ways, such as genetic diversity assessment (Poczai *et al*. 2013).

Specific primers were designed to anneal to conserved parts of common functional genes with the objective of generating polymorphic banding patterns detected afterwards on agarose gels (Poczai *et al*. 2013).

Since there are a large number of conserved gene regions and gene families in plant genomes, any region can be used for this technique. For that reason, Collard and Mackill (2009b) proposed a set of CDDP primers that target well characterized plant genes involved in responses to abiotic stresses. CDDP can generate functional markers related to a given plant phenotype and conserved DNA regions that share the same priming site but differ in their genomic distribution detecting a large number of length polymorphisms (Poczai *et al*. 2013).

CDDPs amplification is based on the use of a single long primer and high annealing temperatures, which improves their reproducibility (Collard and Mackill 2009b; Poczai *et al*. 2013).

CDDP markers have been used for the purpose of genetic diversity assessment and DNA fingerprinting either individually or combined with other markers, such as SCoT (Li *et al.* 2013; Hamidi *et al*. 2014; Hajibarat *et al*. 2015).

1.6. Retrotransposons-based markers

The transposable elements (TEs) were classified into two classes: Class I - “copy-and-paste” retrotransposons (RTNs) with RNA mediated transposition; and Class II - “cut-and-paste” elements (DNA transposons) which mobility is dependent of a DNA intermediate (Sabot *et al.* 2004). According to these authors, the Class I is divided in two subclasses: (i) long terminal repeat (LTR) RTNs that include the autonomous groups *copia*, *gypsy*, and the nonautonomous groups large RTNs derivatives (LARDs) and terminal-repeat RTNs in miniature (TRIMs); and (ii) the non-LTR RTNs that include the Long Interspersed Repetitive Elements (LINEs) and the Short Interspersed Repetitive Elements (SINEs) groups.

The RTNs of Class I are the most abundant in the plant genomes and have LTRs that are highly conserved in their termini, allowing them to be used for primer design and development of RTNs-based markers (Kalendar *et al.* 1999, 2010). Therefore, RTNs-based markers are excellent tools for detecting genetic diversity and genomic changes associated with RTNs activity (Carvalho *et al*. 2010, 2012; Cabo *et al*. 2014b; Fan *et al*. 2014; Delgado *et al*. 2017, among many others).

The new insertional polymorphisms promoted by active members of a RTN family produce polymorphic patterns among genotypes that can be detected after electrophoresis on agarose gels (Kalendar *et al*. 1999, 2010; Kalendar and Schulman 2006; Cabo *et al*. 2014b; Delgado *et al*. 2017, among many others).

RTNs-based markers, such as: the Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) (Kalendar *et al.* 1999; Kalendar and Schulman 2006), and the inter-Priming Binding Site (iPBS) markers (Kalendar *et al*. 2010) screen the whole genome and can detect instabilities and mutational events derived from RTNs activation.

IRAP and REMAP have been used either individually or in combination to study genetic diversity and identify markers related to yield traits due to their generation of reliable and reproducible banding profiles (Carvalho *et al.* 2010, 2012; Poczai *et al*. 2013; Cabo *et al*. 2014b; Jannatdoust *et al*. 2015; Delgado *et al.* 2017, among many others).

The RTNs insertion within the genes can result in mutant alleles that may change the reading frame or the splicing pattern (Makarevitch *et al*. 2015). RTNs can also contribute to the regulation of gene expression, playing an important role in responses to environmental stress (Voronova *et al*. 2014; Makarevitch *et al*. 2015). The transcriptional and transpositional activation of RTNs as response to biotic and abiotic stresses have been widely reported (Grandbastien 1998; Huang *et al*. 2012; Voronova *et al*. 2014, among others). Besides, the RTNs activation has been regarded as mechanism of genotypic remodelling (Fan *et al*. 2014). Therefore, IRAP markers were successfully employed to assess polymorphisms in wheat plants under salt stress (Sigmaz *et al*. 2015). A combination of IRAPs, REMAPs and ISSRs was used to study drought tolerance in wheat (Sen *et al*. 2017). iPBS markers were already used to evaluate the genomic instability of *Pinus sylvestris* plants exposed to heat stress (Voronova *et al*. 2011, 2014).

1.6.1. IRAP markers

IRAP markers were developed by Kalendar *et al*. (1999) and target a group of RTNs that contain direct LTRs. A single or two LTR primers may generate IRAPs by annealing to LTR regions and amplifying the DNA segments between two LTRs (Kalendar *et al*. 1999; Poczai *et al*. 2013) (Fig. 3).

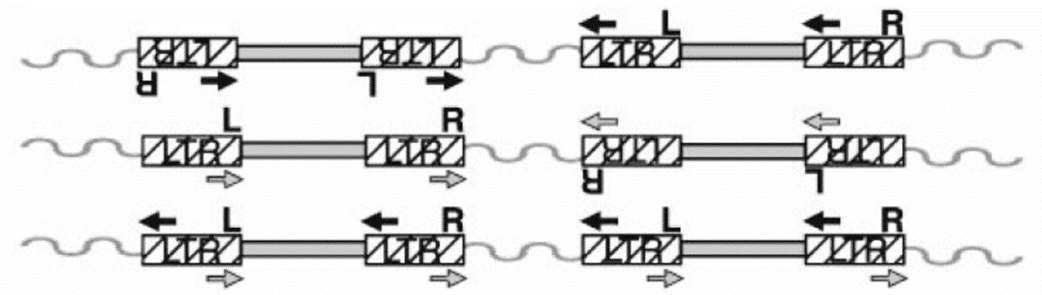


Figure 3. Diagram showing the amplification principle of IRAPs. LTRs (labelled with L or R for left and right, respectively) bounding the internal coding regions (dark box) inserted within flanking DNA (wavy line) where the PCR primers can face outward from the 5' (dark arrows) and 3' (light arrows) ends of the LTRs for the amplification of the intervening DNA in any of the three possible orientations. Adapted from Kalendar *et al.* (1999).

Due to the "copy-paste" method of transposition of the Class I RTNs LTR, the primers can bind to LTR sites in both orientations 5' to 3' or 3' to 5', leading to the differently oriented copy cluster in head-to-head, tail-to-tail or head-to-tail orientation (Poczai *et al.* 2013). For head-to-head and tail-to-tail arrangements, only a single LTR primer is necessary to generate IRAP products, for head-to-tail orientations both 5' and 3' LTR primers are needed to amplify the intervening genomic DNA (Kalendar *et al.* 1999; Poczai *et al*. 2013).

1.6.2. REMAP markers

The REMAP technique exploits polymorphisms in the genomic regions located between a simple sequence repeat (SSR) and a LTR sequence by using a SSR primer and an outward-facing LTR primer, respectively (Kalendar *et al*. 1999; Poczai *et al*. 2013) (Fig. 4).

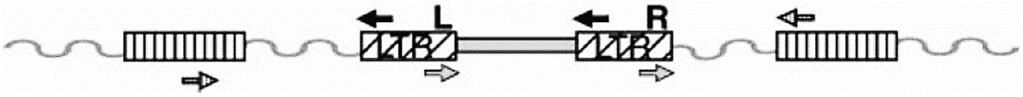


Figure 4. Diagram showing the amplification principle of REMAPs. In a REMAP amplification reaction, a LTR primer similar to those used for IRAP (black and grey arrows) is used together with a SSR primer (segmented arrow) in order to bind to LTR (black box) and SSR (segmented box) regions, respectively, throughout the genome. Adapted from Kalendar *et al*. (1999).

1.6.3. iPBS markers

The development of RTNs-based markers such as IRAP and REMAP in cultivated barley (*Hordeum vulgare* L.) required the knowledge of LTR sequences of the RTN family *BARE-1* (Kalendar *et al.* 1999). Later, it were developed the dominant inter-Priming Binding Site (iPBS) markers which discarded the need of previous knowledge of sequences for primers design, since the highly conserved priming binding site (PBS) sequences among different RTN families were used as primers (Kalendar *et al*. 2010; Poczai *et al*. 2013; Monden *et al*. 2014).

For the amplification of iPBS markers, the RTNs must have opposite direction and must be closer in order to amplify the DNA region between the PBS regions of two RTN LTRs (Kalendar *et al*. 2010; Poczai *et al*. 2013). Therefore, the iPBS amplified product will contain both LTR and PBS sequences together with genomic DNA sequence between the LTRs (Fig. 5).

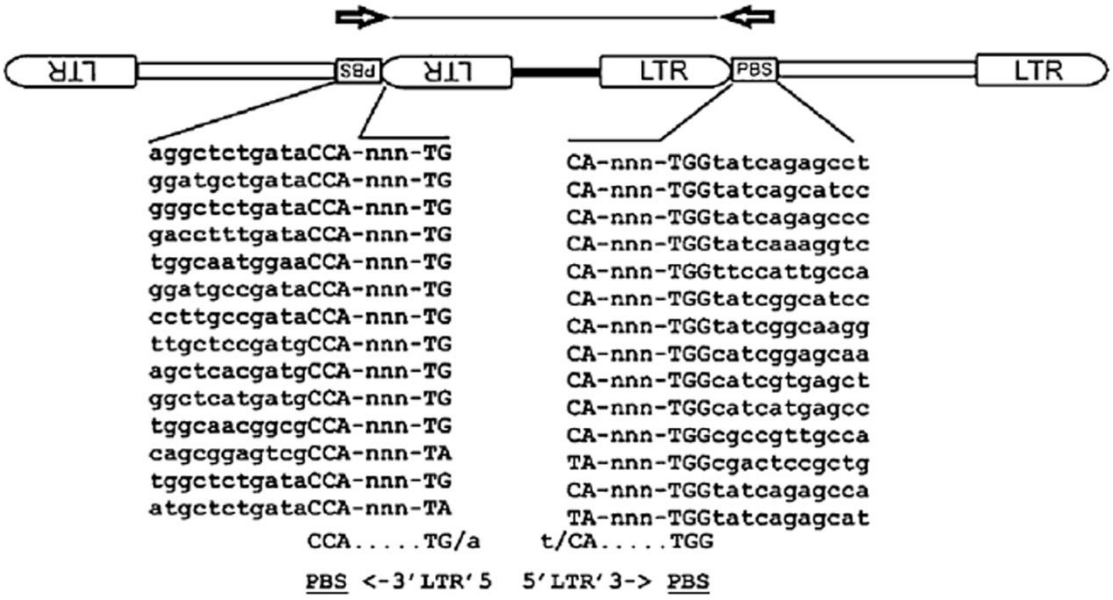


Figure 5. Principle of amplification of iPBS markers. iPBS amplification requires two closer RTNs LTR in opposite orientation. The scheme depicts two key structural features of RTNs, the LTR and PBS regions. The internal domain between two RTNs it is shown as a thick black bar, whereas the intervening amplifiable genomic DNA (predicted product) is presented as a thin black line (above the RTNs LTR structure) with the orientation of the PBS primers (convergent arrows). Below the scheme is presented a set of sequences of PBS domains having flanking regions in CA and TG motifs within the LTR. Source: Kalendar *et al*. (2010).

Since the iPBS amplicons include LTR sequences, these dominant markers can be effective for RTNs isolation and genome scanning (Kalendar *et al.* 2010; Poczai *et al*. 2013).

1.7. ISSR markers

The ISSRs are dominant markers that were developed by Zietkiewicz *et al*. (1994). This technique uses a single SSR primer and consists in the amplification of DNA segments between two close microsatellites or simple sequence repeat (SSR) regions, located in opposite strands and inversely orientated (Fig 6).

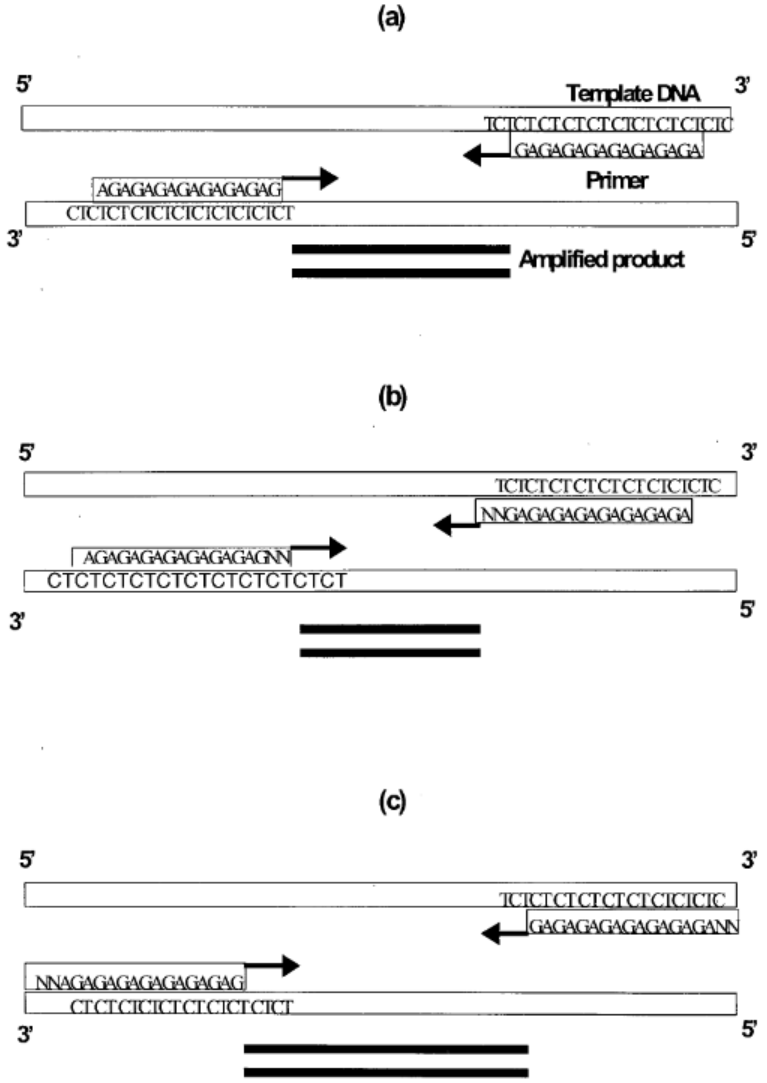


Figure 6. Diagram showing the amplification principle of ISSRs. A single primer $(AG)_8$ unanchored (a), 3'-anchored (b) and 5'-anchored (c) targeting a $(TC)_n$ repeat used to amplify the ISSR region flanked by two inversely oriented $(TC)_n$ sequences. (a) Unanchored $(AG)_n$ primer can anneal anywhere in the $(TC)_n$ repeat region on the template DNA leading to slippage and ultimately smear formation; (b) $(AG)_n$ primer anchored with two degenerated nucleotides (NN) at the 3'-end anneals at specific region on the template DNA, and produces clear bands; (c) $(AG)_n$ primer anchored with two degenerate nucleotides (NN) at the 5'-end anneals at specific regions, amplifying part of the repeat region and leading to larger bands. Source: Reddy *et al.* (2002).

The SSR regions consist of repeat motifs of di-, tri-, tetra- or pentanucleotides that are repeated in tandem, widely spread, abundant and ubiquitous in the eukaryotic genomes (Zietkiewicz *et al*. 1994).The amplification of ISSRs with different SSR primers can give insights about the microsatellite sequences distributed throughout any genome.

The SSRs may respond to abiotic stress through changes in their mutation frequency and stability (Yao and Kovalchuk 2011). In fact, mutant lines of *Arabidopsis thaliana*

subjected to different abiotic stresses showed: SSRs instability; an increase in the homologous recombination frequency (an indicator of genome rearrangements and of stress-induced mutations); point mutations; small deletions and insertions (Yao and Kovalchuk 2011). However, the progenies of *A. thaliana* plants exposed to drought and flood presented mutations in SSRs that were statistically similar to those verified in the progeny of control plants (Yao and Kovalchuck 2011). Besides, other studies have proposed that the transgenerational changes on genome stability and homologous recombination frequency occurs only in the first immediate generation unless the plants continue subjected to the same stress (see review of Boyko and Kovalchuk 2011).

The SSRs stability in plants under drought might justify the successful use of ISSRs for the identification of markers related to drought tolerance in wheat (Deshmukh *et al*. 2012) and other species, such as sugarcane (Patade *et al*. 2005) and sugar beet (Sen and Alikamanoglu 2012), since the genomic rearrangements due to stress may be occurring between SSRs, being detectable with ISSR markers.

1.8. RAPD markers

RAPD markers were developed simultaneously by two groups, Williams *et al*. (1990) and Welsh and McClelland (1990). RAPDs are amplified by the use of a single decamer (10 bp) primer and can detect polymorphism throughout the genome (Fig. 7) without previous knowledge of the specific nucleotide sequence of the target regions to be amplified (Welsh and McClelland 1990).

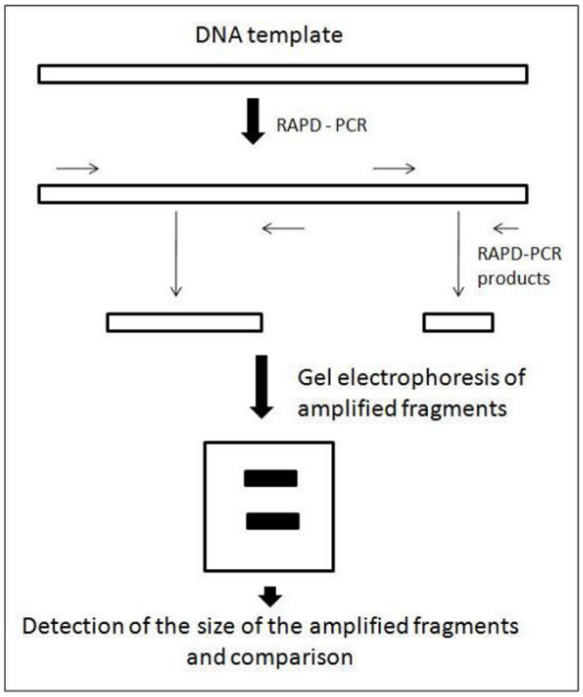


Figure 7. Principle of RAPDs amplification. A single primer (→ or ←) is used per amplification reaction that will bind to complementary sequences in both DNA strands on a convergent sense. Depending on the location of the primer binding sites, the RAPD amplified products (black bands) will present a variable length that could be visualized after electrophoresis on agarose gel. Source: Arif *et al*. (2010).

RAPDs are dominant markers, easy to perform, cost-effective, versatile and allow the fast production of results.

RAPDs enable the detection of DNA polymorphisms caused by insertions or deletions, single base changes due to single mismatches in the primer-genomic DNA duplex preventing the amplification (Welsh and McClelland 1990). Besides, a universal set of primers can be used for genomic analysis in a wide variety of species (Welsh and McClelland, 1990; Liu *et al.* 2007).

Therefore, RAPDs are suited for genetic mapping, DNA fingerprinting of interspecific hybrids, assessment of genetic variability, among other applications (Welsh and McClelland 1990; Lima-Brito *et al.* 2006; among many others).

RAPDs were also used in genotoxicity studies based on the comparison of molecular patterns among individuals untreated (control) and treated with different heavy metals (Cenkci *et al*. 2009; Kekec *et al*. 2010).

The polymorphism detected after the use of micronutrients, heavy metals and other toxic agents is considered an evidence of genomic instability induced by abiotic stress (Atienzar *et al*. 1999; Enan 2007; Liu *et al*. 2007; Cenkci *et al*. 2009; Kekec *et al*. 2010). Hence, RAPD polymorphism was found in *Daphnia magna* treated with benzo[*a*]pyrene (B*a*P), in bean exposed to paranitrophenol (PNP) and Boron (B), as well as in rice treated with Cadmium (Cd) after comparison of their molecular patterns with those achieved in their respective control experiments (Atienzar *et al*. 1999; Enan 2007; Liu *et al*. 2007). Similarly, RAPD polymorphism was detected in bean plants treated with Mercury (Hg), B, Chromium (Cr) or Zn relative to untreated plants (control) (Cenkci *et al*. 2009; Kekec *et al*. 2010). These authors noticed the gain and/or loss of RAPD markers (bands) in treated individuals relative to the control plants. These polymorphic RAPD bands were considered as indicative of genomic instability caused by stress (Cenkci *et al*. 2009; Kekec *et al*. 2010).

The genomic instability evidenced by different DNA markers may arise from different causes: DNA damage (e.g. single and double strand breaks); abasic sites; modified bases; oxidised bases; 8-hydroxyguanine; bulky adducts; point mutations; and/or chromosomal rearrangements (Atienzar and Jha 2006; Liu *et al*. 2007; Cenkci *et al.* 2009). These authors explained the disappearance of bands as a consequence of DNA damage or mutations that cause lesions in the DNA strand altering the PCR kinetics and probable dissociation of the enzyme/adduct complex (Liu *et al*. 2007; Cenkci *et al*. 2009). On the other hand, the newly appeared bands may arise from new annealing events that occur due to mutations within the primer binding sites, to large deletions that bring pre-existing annealing sites closer and to homologous recombination (Liu *et al*. 2007; Cenkci *et al*. 2009)

1.9. Objectives

With this work, we aimed:

- To assess the genomic instability in bread wheat plants of cultivar 'Jordão', under two water availability regimes (watering and drought) after seed priming and/or foliar spraying of AsA or Zn using the gene-targeted markers SCoT and CCDP, the RTNs-based markers IRAP, REMAP and iPBS, as well as ISSRs and RAPDs markers;

- To compare the molecular patterns among untreated (control) and treated plants in each water regime;

- To infer about the most suitable treatment to be applied in bread wheat under drought.

2. MATERIALS AND METHODS

2.1. Plant material

In this work we used seeds and plants of bread wheat (*T. aestivum*) cv. 'Jordão' which it is inscribed on the Portuguese Catalogue of Varieties (CNV 2017). This cultivar has excellent adaptation to Mediterranean conditions, semi-precocious vegetative cycle, great tillering capacity, high productive performance, high baking potential, and high resistance to *Septoria tritici* (leaf blotch), *Puccinia recondita* (brown rust), *Puccinia hordei* (leaf rust) and to other wheat diseases.

The seeds of 'Jordão' wheat were kindly given by José Coutinho, INIAV-Elvas.

2.2. Seed priming treatments

Twenty seeds of 'Jordão' wheat were primed with AsA (2 mM), 20 seeds were primed with 6 mM of Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) and 10 seeds were soaked in distilled water or hydroprimed (control). Seed priming was performed during eight hours at 25 ºC. After priming, the seeds were dried and allowed to germinate in filter paper moistened with distilled water at 25 ºC in the dark for further production of seedlings. The seedlings were installed under greenhouse conditions at the University of Tras-os-Montes and Alto Douro (UTAD) for the further foliar spraying of AsA or $ZnSO_4.7H_2O$ and for the induction of two water availability regimes (watering and drought) during the heading stage.

2.3. Foliar spraying treatments and water availability regimes

Half of the plants (in the greenhouse) whose seeds were primed with AsA or $ZnSO_4.7H_2O$ also received foliar spraying of AsA (1 mM) or $ZnSO_4.7H_2O$ (1.7 mM), respectively.

During the heading phase, two water availability regimes were induced to the AsA- and Zn-treated plants, as well as to the control plants. Half of the plants of each treatment (including control plants) were subjected to (i) induced drought (corresponding to a volumetric value of 5% of water in the soil) during 20 consecutive days, whereas (ii) the other half of the plants (treated and control) were watered and maintained at a good water status (volumetric value of 20% of water in soil) during the same time period. Therefore, two types of control plants were used: control plants in watering and control plants in drought, further named in this work as CW and CD, respectively. The CW plants were compared with watered treated plants, whereas the CD plants were compared to treated plants under drought.

The concentrations of 6 mM $ZnSO_4.7H_2O$ used in seed priming and 1.7 mM $ZnSO_4.7H_2O$ applied as foliar spraying correspond to 0.4% of Zn and 0.1% of Zn, respectively. Therefore, throughout the manuscript the terms Zn priming and/or Zn foliar application will be used for these concentrations/ treatments. Additionally, the concentrations of 2 mM AsA used in seed priming and 1 mM AsA applied as foliar spraying correspond to 0.04% of AsA and 0.02% of AsA respectively. Throughout the manuscript the terms AsA-priming and/or AsA foliar spraying will refer to these concentrations and treatments.

2.4. Genomic DNA extraction

Leaves of 50 bread wheat plants (control and treated) were collected at the end of the drought induction, immediately frozen in liquid nitrogen and maintained at -80 ºC till genomic DNA extraction using a CTAB-based method (Doyle and Doyle 1987).

The DNA samples were quantified in the spectrophotometer Nanodrop® ND-1000 (Thermo Scientific) and their integrity was evaluated after electrophoresis on 0.8% agarose gel. Working solutions with 25 ng/µL of concentration were prepared.

2.5. Primers tested for SCoT and CDDP amplifications

The 36 SCoT primers previously developed by Collard and Mackill (2009a) were individually tested.

For the amplification of CDDP markers, we individually tested the forward and reverse primers of stress-responsive genes such as three WRKY transcription factors and the R2-R3 protein-like Myb gene *PIMP1* of *T. aestivum*, previously isolated and characterized by others (Liu *et al*. 2011; Wang *et al*. 2015; He *et al*. 2016) (Table 1).

Table 1. Stress-responsive gene, respective GenBank accession, reference and sequences of the primers individually tested for CDDPs amplification.

Gene	**GenBank accession**	**Reference**	**Sequence 5'→3'**
TaWRKY1	KT285206	He *et al*. 2016	F: ATATGGCGGCACTTGTCACT
			R: CAGAGGAATGGCGTCAAAAT
TaWRKY33	KT285207		F: GAGGTTGCGGTTCTTGAGTC
			R: AGGTTCCGACGGATCATCT
TaWRKY44	KR827395	Wang *et al*. 2015	F: GCCCCCCTTCGCTCTTCTC
			R: CAGCACACCAGAAATGGGCTAAT
TaPIMP1 (Myb gene)	EF587267	Liu *et al*. 2011	MQC-F: ACTCGCGTACGTCTTCCTGA
			MQC-R: GCGCTCTAGTTAAGTTCATCGTC

We used the primers previously reported by Wang *et al*. (2015) and Liu *et al*. (2011) for the genes *TaWRKY44* and *TaPIMP1*, respectively. Additionally, we designed specific primers for the sequences of genes *TaWRKY1* and *TaWRKY33*, publicly available in GenBank (Table 2), using the software Primer3 (v. 0.4.0) (Koressaar and Remm 2007; Untergrasser *et al*. 2012), and the following criteria: primer length of 18 to 20 base pair (bp), GC content of 45 to 60%, and melting temperature (Tm) of 60 ºC.

2.6. Primers tested and selected for IRAP, REMAP, ISSR and iPBS amplifications

In this study, we tested individually LTR, SSR and PBS primers that were designed and previously used by other authors to amplify IRAP, REMAP, ISSR and iPBS markers in different plant species, including wheat (Table 2).

Table 2. LTR, SSR and PBS primers individually tested, respective references and sequences.

Primers		**Reference**	**Sequence 5'→3'**
LTR	*5'LTR BARE-1*	Saeidi *et al*. (2008)	ATCATTGCCTCTAGGGCATAATTC
	6149	Kalendar *et al*. (1999)	CTCGCTCGCCCACTACATCAACCGCGTTTATT
	6150	Kalendar *et al*. (1999)	CTGGTTCGGCCCATGTCTATGTATCCACACATGGTA
	Nikita	Shirasu *et al*. (2000)	CGCATTTGTTCAAGCCTAAACC
	Sukkula	Bento *et al*. (2008)	GATAGGGTCGCATCTTGGGCGTGAC
	7286	Kalendar *et al*. (1999)	GGAATTCATAGCATGGATAATAAACGATTATC
	Sabrina	Bento *et al*. (2008)	GCAAGCTTCCGTTTCCGC
	Tagermina	Queen *et al*. (2004)	AGAGGAGGATATCCCAACAT
	Thv 19	Queen *et al*. (2004)	GCCCAACCGACCAGGTTGTTACAG
	Tar 1	Queen *et al*. (2004)	CTCCCAGTTGACCAACAA
SSR	8081	Kalendar *et al*. (1999)	GAGAGAGAGAGAGAGAGAC
	8082	Kalendar *et al*. (1999)	CTCTCTCTCTCTCTCTCTG
	8564	Kalendar *et al*. (1999)	CACCACCACCACCACCACCACT
PBS	*F0100*	Wegscheider *et al*. (2009)	TAGGTCGGAACAGGCTCTGATACCA
	2222	Kalendar *et al*. (2010)	ACTTGGATGCCGATACCA
	2224	Kalendar *et al*. (2010)	ATCCTGGCAATGGAACCA
	2228	Kalendar *et al*. (2010)	CATTGGCTCTTGATACCA
	2229	Kalendar *et al*. (2010)	CGACCTGTTCTGATACCA
	2230	Kalendar *et al*. (2010)	TCTAGGCGTCTGATACCA

Based on the primers that showed successful amplification, we select combinations that were previously fruitful in cereals in order to produce additional RTNs-based markers (Table 3).

Table 3. Combinations of primers selected in this work and which were previously successful in other studies performed in cereals.

Marker	Primer Combination	Reference
IRAP	*5'LTR BARE-1* + *Sukkula*	Carvalho *et al*. (2010, 2012)
	6149 + *Sukkula*	Carvalho *et al*. (2010, 2012)
	6150 + *Sukkula*	Carvalho *et al*. (2010, 2012)
	Nikita + *Sukkula*	Carvalho *et al*. (2010, 2012)
	Sukkula	Bento *et al*. (2008); Carvalho *et al*. (2010, 2012); Cabo *et al*. (2014b)
REMAP	7286 + 8081	Carvalho *et al*. (2010, 2012)
	Nikita + 8081	Carvalho *et al*. (2010, 2012)
	Sukkula + 8081	Carvalho *et al*. (2010, 2012); Cabo *et al*. 2014b
ISSR	8081	Kalendar *et al*. (1999); Carvalho *et al*. (2010, 2012); Cabo *et al*. (2014b)
iPBS	*F0100*	Wegscheider *et al*. (2009); Cabo *et al*. (2014b)

2.7. Primers tested for RAPDs amplifications

A total of 13 RAPD primers, one from the kit A and 12 from the kit B of the Operon Technologies (Qiagen) (Table 4) were tested. These primers were previously used by other authors for detection of genomic instability in bean and wheat under abiotic stress (Cenkci *et al*. 2009; Kekec *et al*. 2010) (Table 4).

Table 4. RAPD primers tested in this work and respective sequences.

Primer name	Sequence 5'→3'
OPA08	CCACAGCAGT
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPB06	TGCTCTGCCC
OPB07	GGTGACGCAG
OPB08	GTCCACACGG
OPB09	TGGGGGACTC
OPB10	CTGCTGGGAC
OPB11	GTAGACCCGT
OPB12	CCTTGACGCA
OPB13	TTCCCCCGCT

2.*8. Amplification of SCoT and CDDP markers*

The PCR mixture and the visualization of SCoT markers followed those described by Cabo *et al*. (2014a).

The PCR mixture used for amplification of CDDP markers (final volume of 10 μL) was adapted from that described by Collard and Mackill (2009b) and it was composed by: 25 ng of genomic DNA; 1x PCR buffer with KCl; 1.5 mM of $MgCl_2$; 0.25 mM of each dNTP; 1.6 μM of primer; and 0.2 U of *Taq* DNA polymerase (Thermo Scientific).

The conditions of amplification of CDDP markers were the same that were used to SCoTs (Collard and Mackill 2009a; Cabo *et al*. 2014a). The CDDP amplification products were visualized after electrophoresis on 2% agarose gels prepared with 1x TBE buffer and stained with ethidium bromide.

All PCR reactions were repeated at least twice to verify the reproducibility of all bands, on different days and thermal cyclers as recommended by Collard and Mackill (2009a).

2.9. Amplification of IRAP, REMAP, ISSR and iPBS markers

The PCR mixture used for amplification of IRAP, REMAP and ISSR markers (final volume of 20 µL) was composed by: 50 ng of genomic DNA, 1x PCR buffer with $(NH_4)_2SO_4$, final concentration of 1.5 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.625 µM of primer and 0.1 U of *Taq* DNA polymerase (Thermo Scientific).

For iPBS markers amplification we used a PCR mixture (final volume of 20 µL) composed by: 75 ng of genomic DNA, 1x PCR buffer with $(NH_4)_2SO_4$, final concentration of 1.5 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.5 µM of primer and 0.2 U of *Taq* DNA polymerase (Thermo Scientific).

The amplification conditions consisted in: an initial denaturation at 94 ºC for 4 min followed by 30 cycles, each one composed by denaturation at 94 ºC for 1 min, annealing at 53 ºC during 1 min and extension at 72 ºC for 2 min and 30 sec; and a final extension at 72 ºC during 10 min.

The iPBS amplified products were visualized after electrophoresis on 2% agarose gels prepared with 1x TBE buffer and stained with ethidium bromide.

All PCR reactions were repeated at least twice to verify the reproducibility of all bands.

2.10. Amplification of RAPD markers

The PCR mixture used for amplification of RAPD markers (final volume of 25 µL) was composed by: 62.5 ng of genomic DNA, 1x PCR buffer with $(NH_4)_2SO_4$, final concentration of 2.5 mM of $MgCl_2$, 0.05 mM of each dNTP, 0.4 µM of primer and 0.16 U of *Taq* DNA polymerase (Thermo Scientific).

The amplification conditions consisted in: an initial denaturation at 94 ºC for 5 min followed by 44 cycles, each one composed by denaturation at 94 ºC for 1 min, annealing at 37 ºC during 1 min and extension at 72 ºC for 2 min; and a final extension at 72 ºC during 5 min.

The RAPD amplified products were visualized after electrophoresis on 2% agarose gels prepared with 1x TBE buffer and stained with ethidium bromide.

All PCR reactions were repeated at least twice to verify the reproducibility of all bands.

2.11. Analysis of the molecular markers

During this work, AsA- and Zn-treated plants under drought and watering regimes were characterized with different molecular marker systems and their molecular profiles were compared with those achieved in the control plants of both water regimes.

In each agarose gel, the molecular weight marker Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific) was loaded.

Each band was considered a SCoT, CDDP, IRAP, REMAP, ISSR, iPBS or RAPD marker. Bands with the same molecular weight produced by the same primer were considered the same *locus*.

The bands were analysed for their presence (1) or absence (0) among treated and control plants of each water availability regime (watering and drought) for further construction of binary matrices. Additionally, within each water regime the AsA- and Zn-treated plants were separately analysed and compared with their respective control plants.

Since ISSRs and IRAPs are amplified with a single SSR and LTR primer, respectively, REMAP bands with an equal molecular weight to ISSRs and/or IRAPs produced with the same primer were discarded from the REMAP matrix, in order to ensure the effective analysis of REMAP markers.

SCoT, CDDP, IRAP, REMAP, ISSR, iPBS and RAPD polymorphism in treated plants (plants present on both water regimes and treated with Zn or AsA) comprised the disappearance of a band present in the control plants (‘normal band’) and/or the appearance of a new band relatively to the patterns observed in the control plants.

Since polymorphic patterns were produced among the control plants of the watering and drought regimes with all marker systems, only the monomorphic bands among the control plants of each water regime, named as ‘normal bands’, were considered for the comparative analyses of molecular patterns.

For each marker system, the percentage of polymorphism (%P) was calculated per primer, treatment and water regime, with the formula: %P = [number of polymorphic bands

(P)/ total number of amplified bands (T) x 100%], considering as polymorphic bands the lost and new bands, and as total amplified bands the 'normal bands' of the control plants.

2.12. Statistical analyses

2.12.1. Correlation Mantel tests

A total of 14 binary matrices produced as result of the presence/ absence analysis of bands produced by each marker system in AsA- and Zn-treated individuals and their respective controls, were converted to genetic distance matrices using the Nei's genetic distance coefficient (Nei 1987) and the software GenalEx 6.0 (Peakall and Smouse 2006).

The genetic distance matrices of each pair of marker systems were compared through the correlation Mantel test (Smouse *et al*. 1986) performed with 999 permutations and a significance level of 95%, using the software GenalEx 6.0 (Peakall and Smouse 2006).

The pairs of marker systems were defined within and accordingly their category of classification of molecular markers as proposed by Poczai *et al*. (2013), namely: conserved DNA based markers and targeted fingerprinting based markers (CDDPs *versus* SCoTs); transposable elements based markers (IRAP *versus* REMAP, IRAP *versus* iPBS and REMAP *versus* iPBS) and arbitrarily amplified DNA markers (ISSRs *versus* RAPDs). However, for a question of simplicity, throughout this work, the former two types will be named as gene-targeted markers for SCoTs and CDDPs, and RTNs-based markers for IRAP, REMAP and iPBS.

Pairs of marker systems showing a reduced correlation coefficient ($r < 0.900$) between their genetic distance matrices were analysed separately.

2.12.2. Analyses of variance

Analyses of variance (ANOVA) and the post hoc Fisher's Protected Least Significant Difference (PLSD) test based on the total number of lost and new SCoT, CDDP, IRAP, REMAP, ISSR, iPBS and RAPD bands, determined per treatment and water availability regime, were performed with the software StatView 5.0 (SAS Institute Inc. Copyright). The Equality of Variances F test was also performed with the same software. The *F* values

significance, due to the different variable or effects and their interaction, was established for a probability lower than 5% ($p < 0.05$).

2.12.3. Principal Coordinates Analysis of the molecular data

Genetic distance matrices based on the pooled molecular data achieved with each marker system in AsA- and Zn-treated plants, were calculated with the Nei's genetic distance coefficient (Nei 1987) and the software GenalEx 6.0 (Peakall and Smouse 2006).

Principal Coordinates Analyses (PCoAs) were performed based on the genetic distance matrices in order to elucidate the total or cumulative percentage of molecular variation explained by the sum of the values of the first three coordinates or axes, and to analyse the projection of the individuals under study.

3. RESULTS

3.1. Polymorphic patterns among control plants of each water regime

Figure 8 presents ISSR and RAPD polymorphic patterns observed among the control plants of each water regime. Such feature was also observed with the remaining marker systems used in this work.

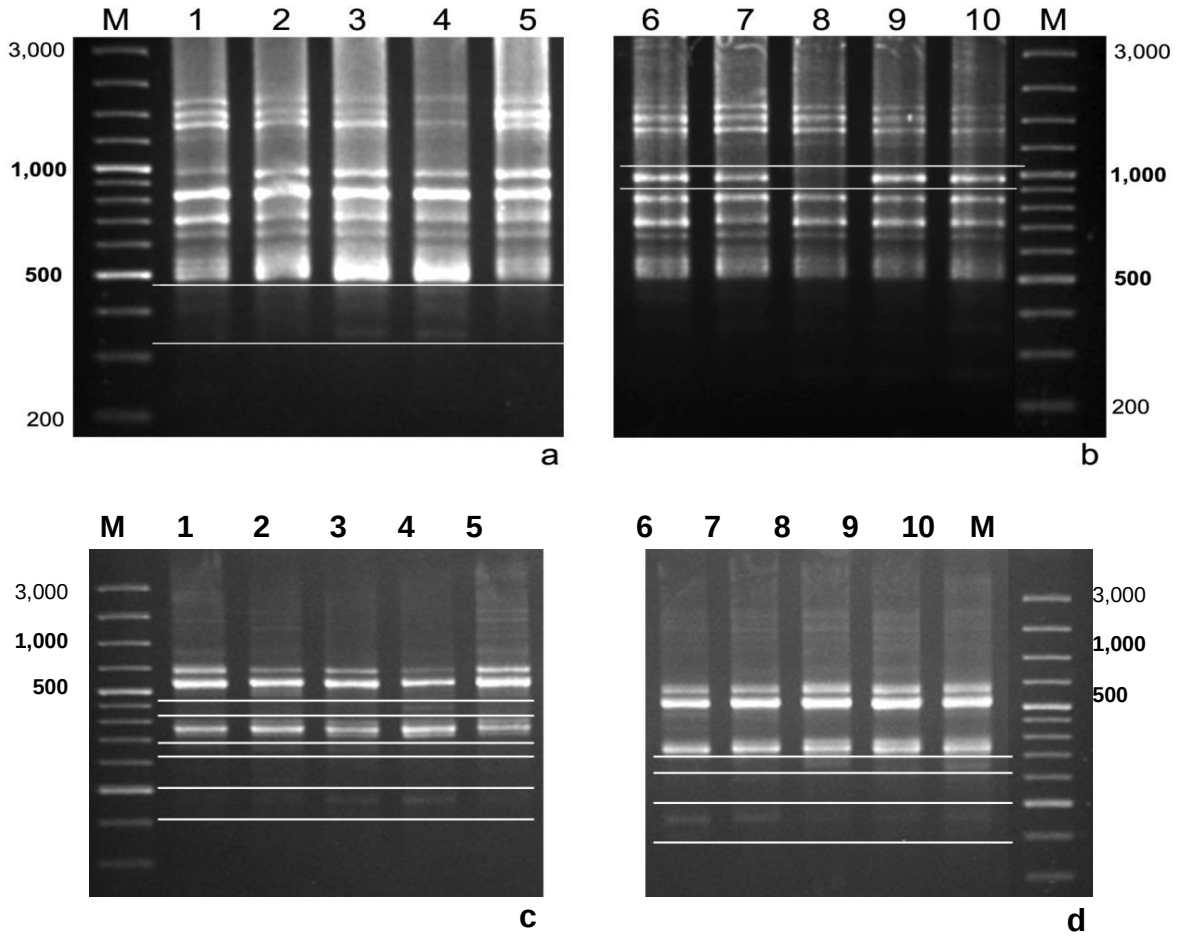


Figure 8. ISSRs produced with the SSR primer 8081 (a, b) and RAPDs amplified with primer OPB13 (c, d) showing polymorphic bands (between the white lines) among the control plants of: (a, c) the watering (lanes 1 to 5) and (b, d) drought (lanes 6 to 10) regimes. M – Molecular weight marker.

3.2. Correlation of the molecular data achieved in AsA- and Zn-treatments

In this work, seven distinct marker systems were used. Accordingly the classification proposed by Poczai *et al*. (2013), the used marker systems can be categorized into: (i) conserved DNA based markers and targeted fingerprinting markers (SCoT and CDDP); (ii) transposable elements (TEs) or RTNs-based markers (IRAP, REMAP and iPBS); and (iii) arbitrarily amplified DNA markers (ISSRs and RAPDs), Hence, in order to verify if we can combine molecular data produced by marker systems of the same category in the further analyses, different correlation Mantel tests were performed.

3.2.1. Correlation Mantel test between CDDP and SCoT molecular data

Genetic distance matrices based on the pooled CDDP and pooled SCoT data produced in the AsA- and Zn-treated plants of both water regimes and respective controls were constructed. Two correlation Mantel tests were performed between the pooled CDDP and SCoT data achieved in the AsA- and in the Zn-treated plants (Fig. 9).

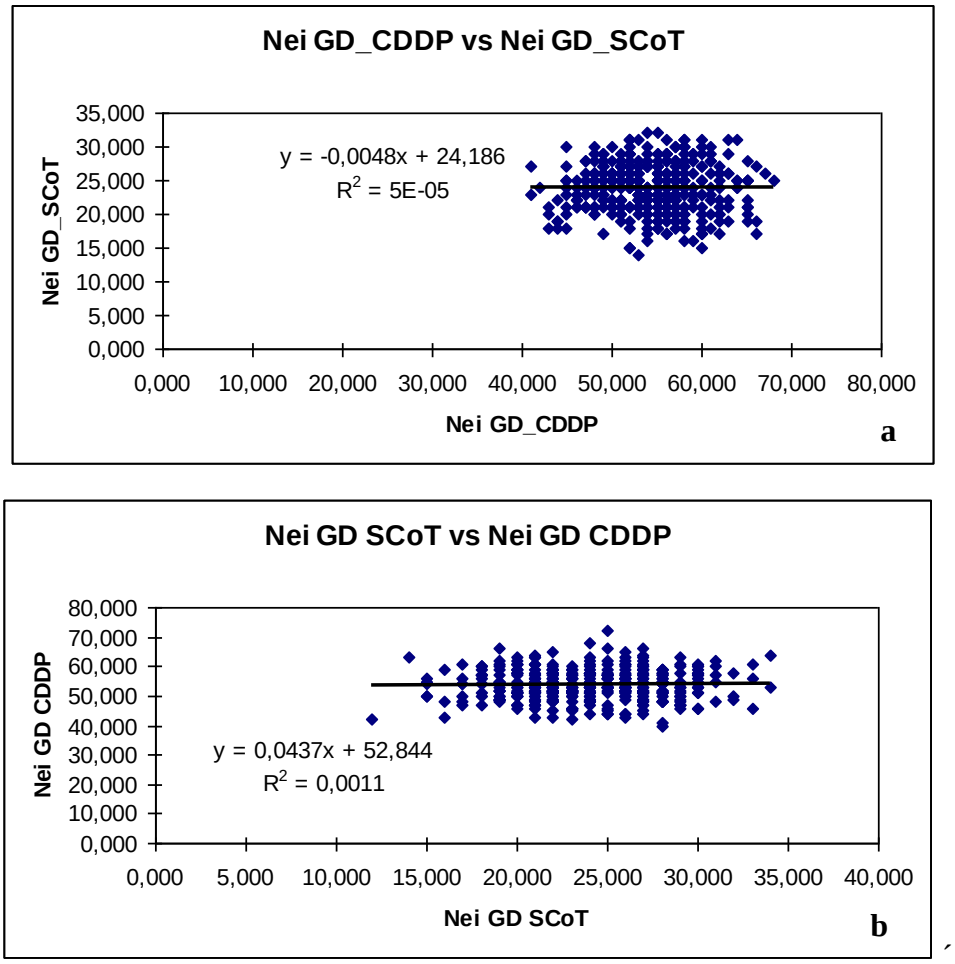


Figure 9. Correlation Mantel tests between the genetic distance matrices based on the pooled CDDP and SCoT data achieved in Asa- (a) and Zn-treated (b) plants of both water availability regimes and respective controls.

The correlation coefficient (*r*) between the genetic distance matrices constructed based on the pooled CDDP and SCoT data achieved in AsA-treated plants of both water regimes and their controls was almost null (*r* = 0.007), indicating that there was no relationship between the two matrices (Fig. 9-a). A *p*-value = 0.456 was achieved, revealing that the genetic distance among the analysed AsA-treated individuals were not statistically significant ($p > 0.05$). Similarly, in Zn-treated plants the Mantel test also revealed a reduced correlation coefficient (*r* = 0.033) (Fig. 9-b). The genetic distance among the Zn-treated plants of both water regimes and their controls was no statistically significant (*p* = 0.237).

Based on the Mantel tests performed between the genetic distance matrices of CDDP and SCoT data, in the following analyses the molecular data achieved in the AsA and Zn-treatments with these two marker systems are presented separately.

3.2.2. Correlation Mantel test among the molecular data produced with the three RTNs-based marker systems

Three genetic distance matrices based on pooled data achieved with the three RTNs-based marker systems IRAP, REMAP and iPBS in the AsA-treated plants and respective controls of both water regimes were constructed. These matrices were compared in the possible combinations by using the correlation Mantel test (Fig. 10).

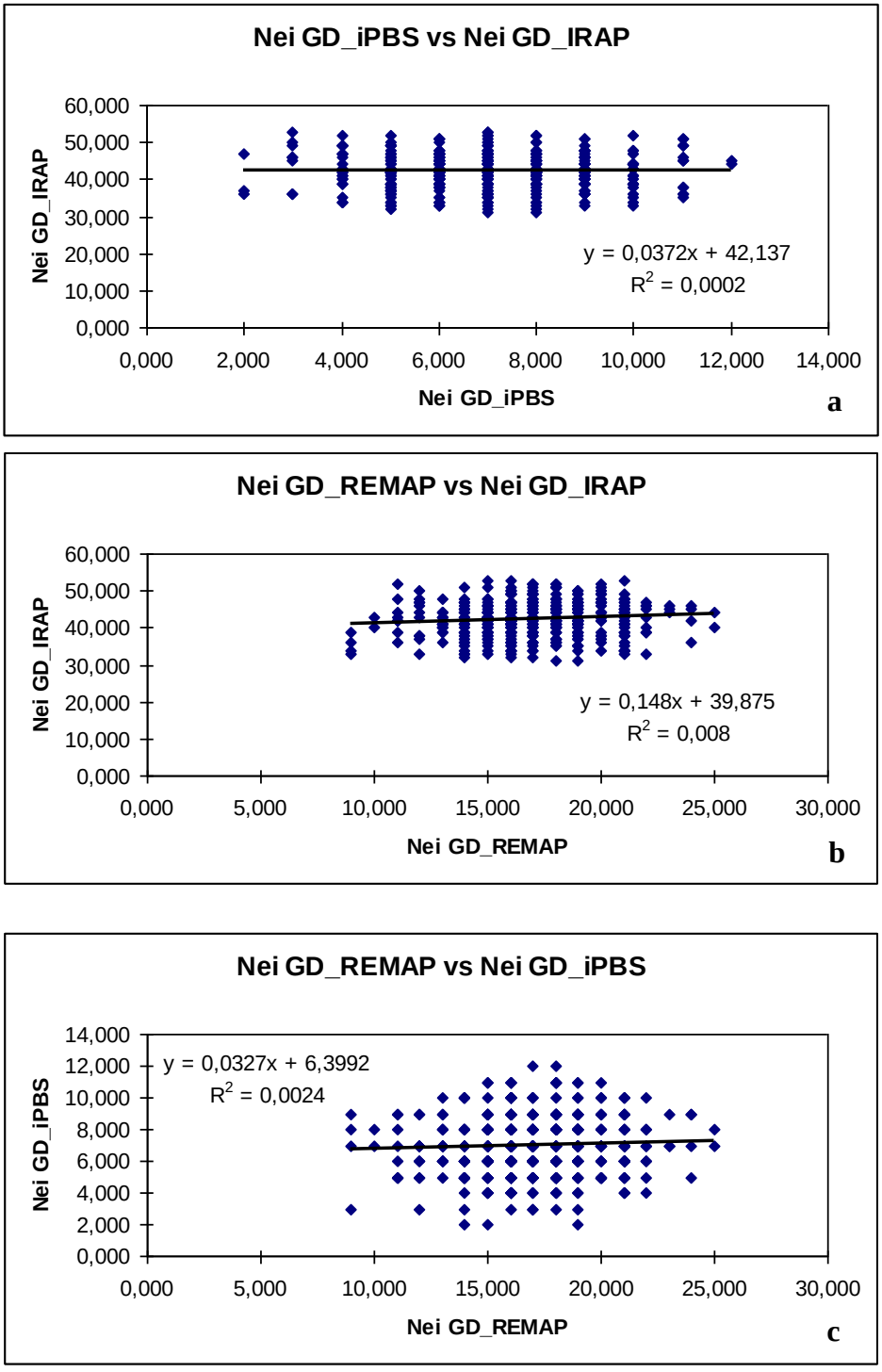
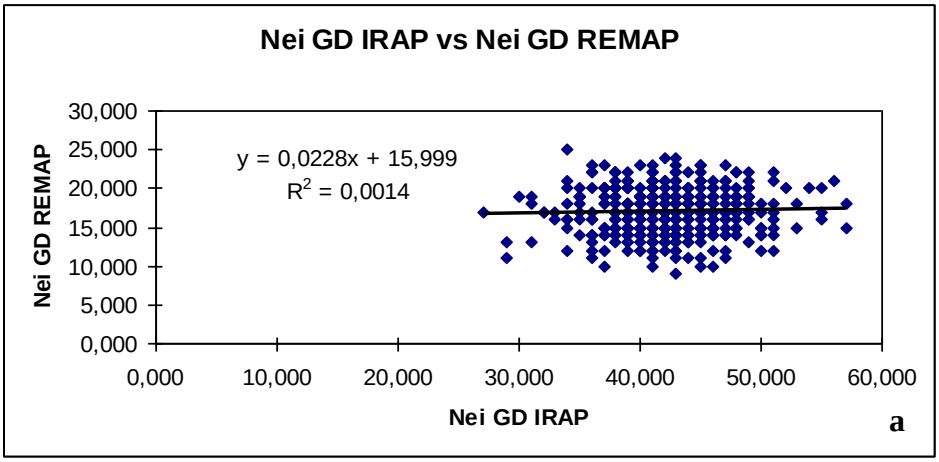
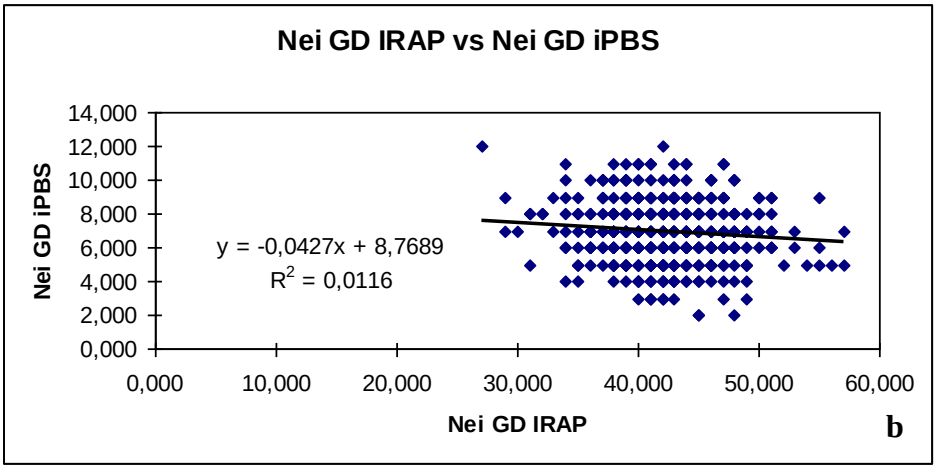


Figure 10. Correlation Mantel tests performed between the genetic distance matrices based on the pooled (a) REMAP and IRAP data (r = 0.09 and p = 0.039); (b) iPBS and IRAP (r = 0.014 and p = 0.397); and (c) REMAP and iPBS data (r = 0.049 and p = 0.151), achieved in plants of both water availability regimes treated with AsA and respective controls.

The Mantel tests graphically presented in Figure 10 revealed in all cases a reduced correlation coefficient (*r*), between the analysed genetic distance matrices. Regarding the *p*-values, only that calculated between the genetic distance matrices based on the pooled IRAP and REMAP data was statistically significant ($p = 0.039$) (Fig. 10), suggesting significant differences between the genetic distances assessed by these markers. Therefore, the IRAP and REMAP data obtained in AsA-treated plants will be also analysed separately.

Figure 11 shows the results of the correlation Mantel tests between each pair of genetic distance matrices generated with the three RTNs-based markers in the Zn-treated and control plants of both water regimes.





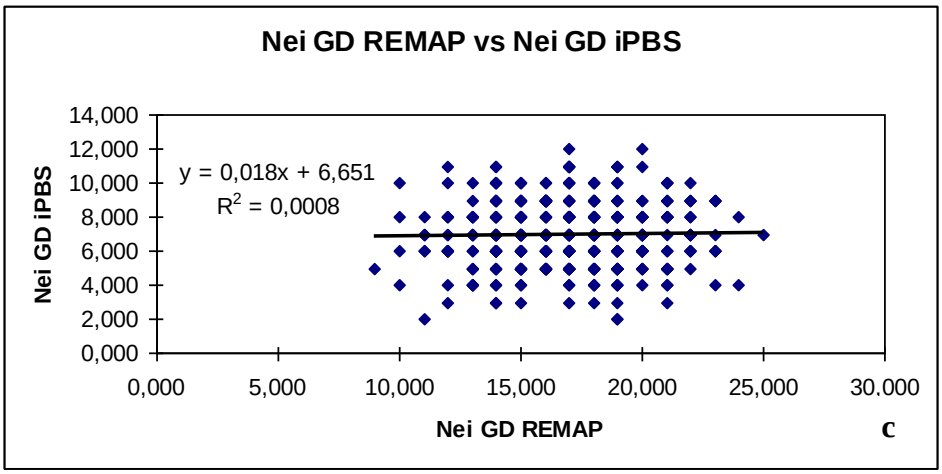


Figure 11. Correlation Mantel tests performed between the genetic distance matrices based on the pooled (a) IRAP and REMAP data (r = 0.037 and p = 0.234); (b) IRAP and iPBS (r = 0.108 and p = 0.016); and (c) REMAP and iPBS data (r = 0.028 and p = 0.265), achieved in plants of both water availability regimes treated with AsA and respective controls.

The results of the correlation Mantel tests graphically represented in Fig. 11 revealed a reduced correlation coefficients (*r*) between each pair of genetic distance matrices generated with the RTNs-based markers in the Zn-treated plants of both water regimes and respective controls. Regarding the *p*-values, only that calculated for the Mantel test between IRAP and iPBS was statistically significant ($p = 0.016$) (Fig. 11).

Overall, the molecular data produced with IRAP, REMAP and iPBS in Zn-treated and control plants of both water regimes will be analysed separately.

3.2.3. Correlation Mantel test between ISSR and RAPD markers

In this study, the ISSR markers were used for the effective identification of REMAP markers. However, since ISSRs are arbitrarily amplified markers like RAPDs, we performed two correlation Mantel tests between the ISSR and RAPD genetic distance matrices obtained in the AsA- and in the Zn-treated plants in order to verify if it was possible to combine them (Fig. 12).

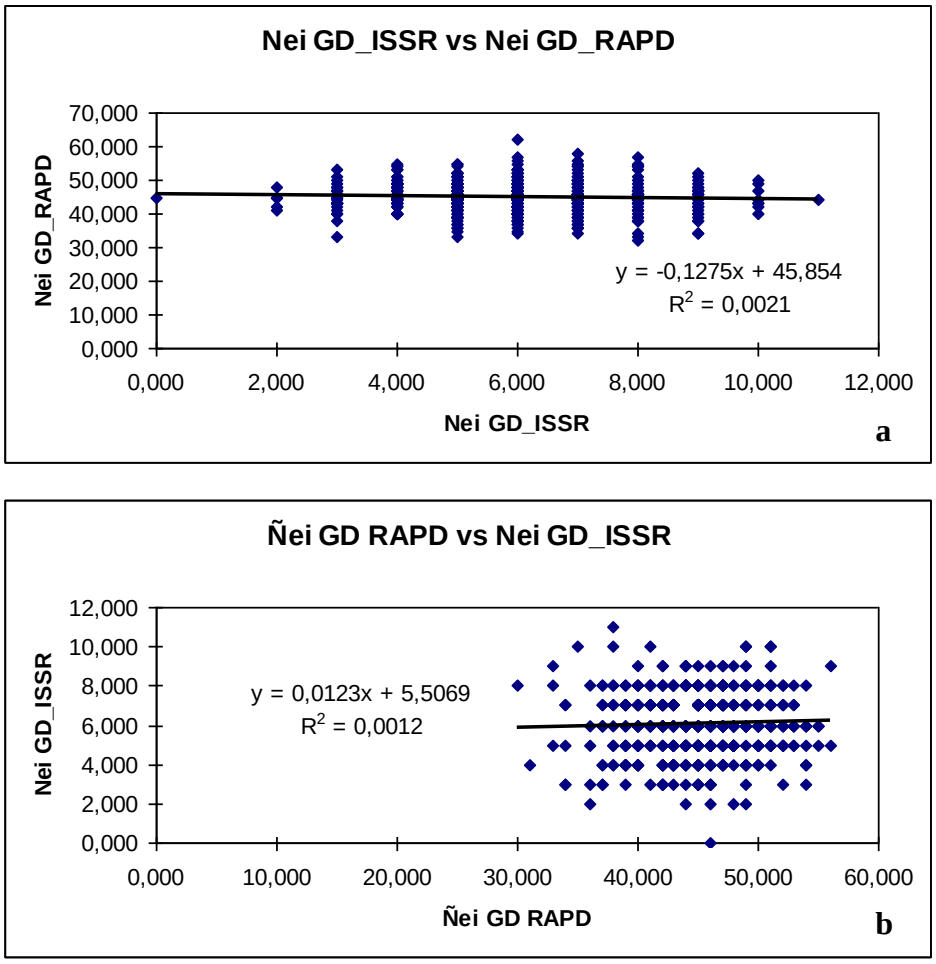


Figure 12. Correlation Mantel tests performed between the genetic distance matrices based on the pooled ISSR and RAPD data achieved in plants of both water availability regimes treated with: (a) AsA (r = 0.046 and p = 0.168) and (b) Zn (r = 0.035 and p = 0.244), and respective controls.

The correlation coefficient (r) between the ISSR and RAPD genetic distance matrices was low in both AsA- and Zn-treatments (Fig. 12). In both treatments, the p-values revealed no statistically significant differences ($p > 0.05$) between the genetic distances calculated with ISSRs and RAPDs (Fig. 12). However, due to the reduced r-values, these molecular data were also analysed separately in the further sections.

Overall, based on the correlation Mantel tests performed, it was decided to analyse the molecular data produced by each marker system separately in the following analyses.

3.2.4. Evaluation and comparison of molecular patterns

The results achieved with the comparison of the molecular patterns among the treated plants of each water regime and respective controls, and the subsequent statistical analyses based on the sum of lost and new bands detected in treated plants relative to control, produced by the different molecular marker systems are presented in the following items. In addition, the AsA- and the Zn-treatments were analysed separately.

3.2.5. Polymorphism among treated and control plants evidenced by gene-targeted markers

Here we tested 36 SCoT primers previously developed by Collard and Mackill (2009a). However, only five primers showed successful amplification and/or produced polymorphic SCoT patterns among treated and control plants of both water regimes (Tables 5 and 7).

Among the eight CDDP primers tested, seven showed successful amplification and produced polymorphic patterns among the control and treated plants of both water regimes (Tables 5 and 7).

Due to the occurrence of polymorphism among the control plants of both water regimes, it were considered ‘normal bands’ those that were monomorphic among the control individuals of each water regime, in order to proceed with the comparison of molecular markers among treated and control plants.

Figures 13 and 14 represent SCoT and CDDP polymorphic patterns, respectively, that were detected in AsA- and Zn-treated plants of both water availability regimes after comparison with their respective controls.

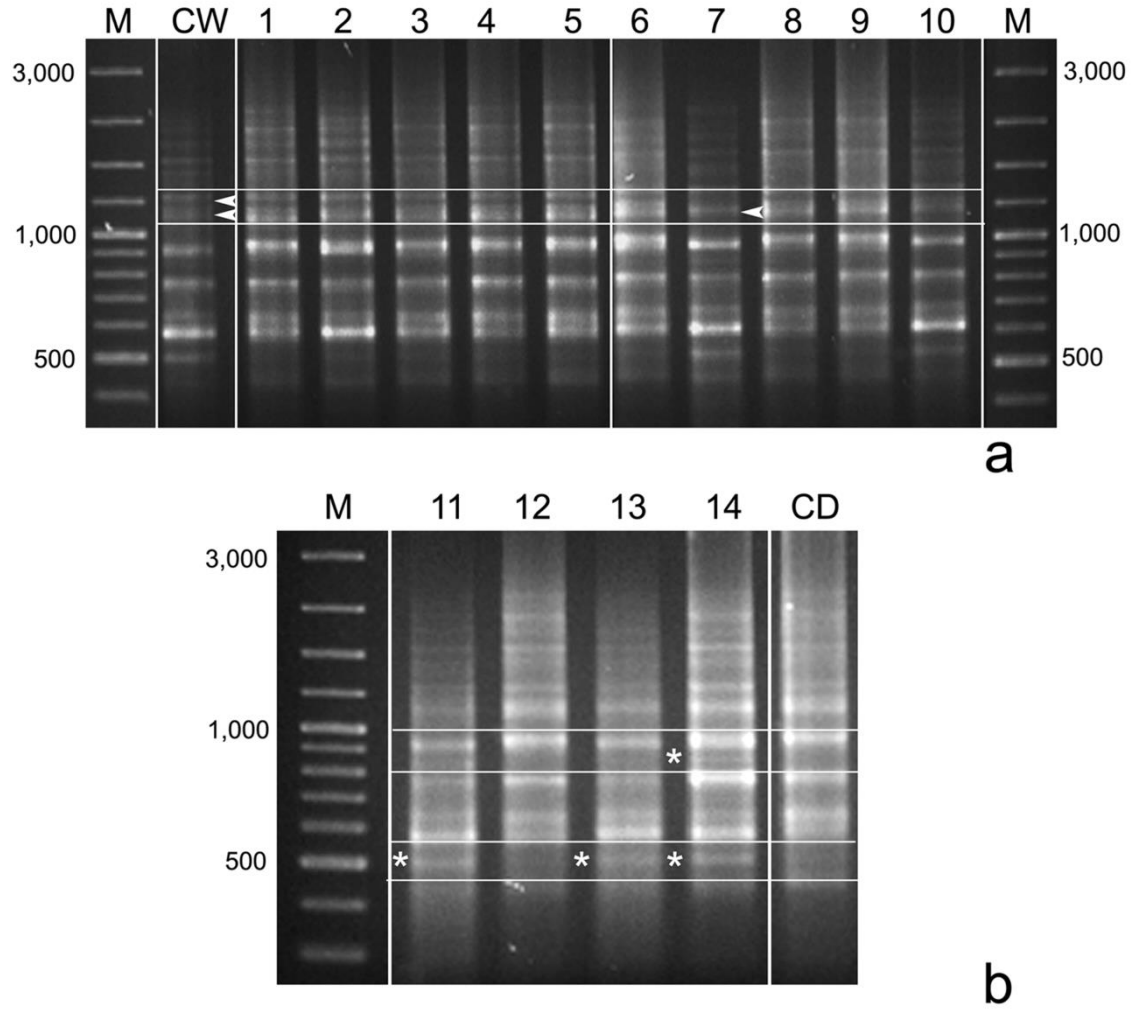


Figure 13. SCoT markers amplified with primer SCoT 7 in plants of the watering (a) and drought (b) regimes. The watered plants treated with Zn priming (lanes 2 to 5) presented similar patterns with the control in watering (CW). Among the watered plants treated with Zn priming plus foliar spraying (lanes 6 to 10), one of them (lane 7) lost one SCoT marker (arrow) relative to the control (CW). In the drought regime (b), three individuals treated with AsA priming plus foliar application (lanes 11, 12 and 13) showed one or two new SCoT bands (*) relative to the control in drought (CD). M – Molecular weight marker.

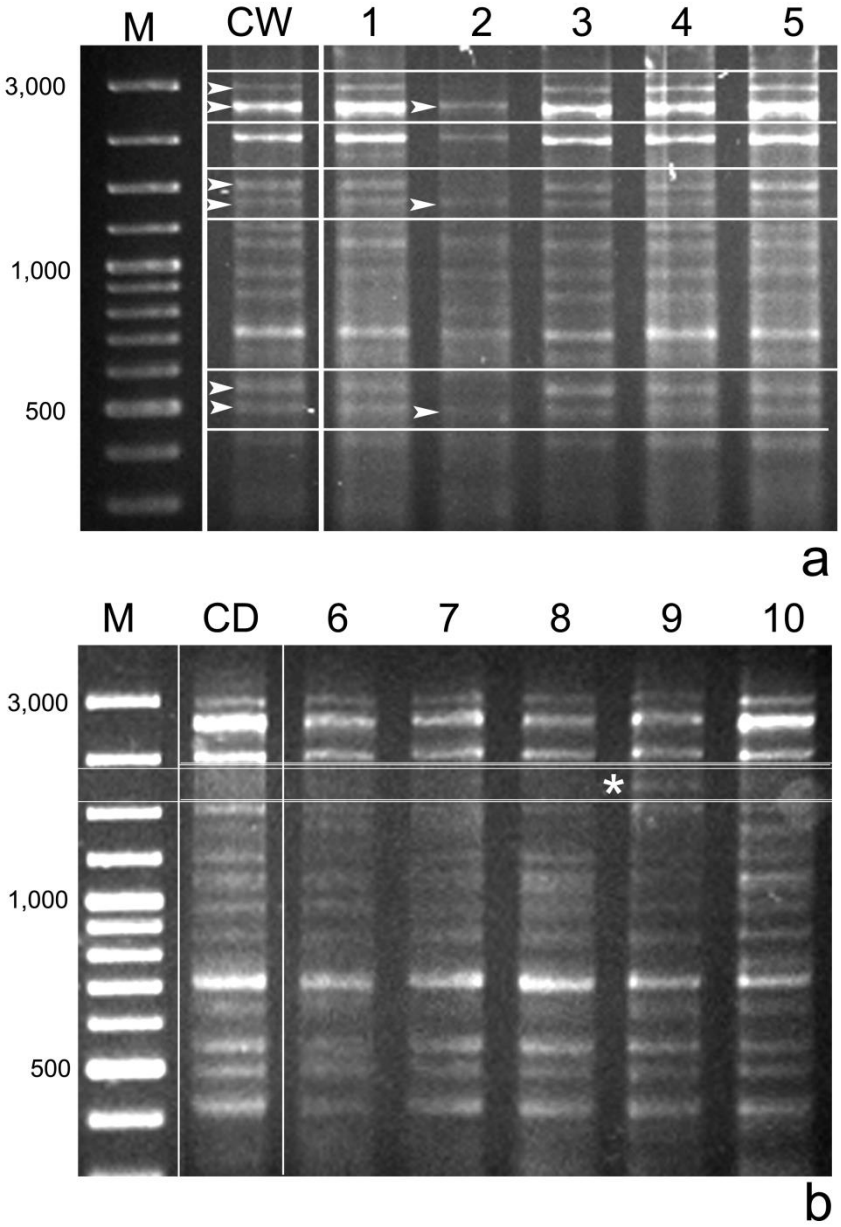


Figure 14. CDDP markers amplified with primer MQC-F in plants under watering (a) and drought (b) regimes. (a) Individuals from lanes 1 to 5 were treated with Zn priming and one individual (lane 2) lost three CDDP markers (arrows) relative to the watering control (CW). (b) Among the plants under drought and treated with Zn priming (lanes 6 to 10), one (lane 8) showed a new CDDP marker (*) relative to the control in drought (CD). M – Molecular weight marker.

3.2.5.1. AsA-treated and control plants characterized by gene-targeted markers

Table 5 presents the number and molecular size of new and lost CDDP and SCoT bands detected in AsA-treated plants by comparison with the control plants in both water regimes.

Table 5. Molecular size (base pair, bp) and number of newly appeared (+) and disappeared (-) SCoT and CDDP bands relative to control plants detected per primer in AsA-treated plants of both water regimes.

Marker/ Primer		Watering		Drought	
		AsA priming	AsA priming and foliar application	AsA priming	AsA priming and foliar application
SCoT					
SCoT 1	+	-	-	-	-
	-	500 bp	500 bp	-	1000 bp
SCoT 2	+	-	-	-	-
	-	-	1100; 1000 bp	2300; 1700; 1000; 600 bp	1000; 600 bp
SCoT 4	+	-	-	-	-
	-	900 bp	900 bp	2550; 2100; 700 bp	-
SCoT 7	+	-	-	2300 bp	900; 500 bp
	-	-	500 bp	-	-
SCoT 11	+	-	-	-	-
	-	-	2250; 2000 bp	500 bp	2250; 500 bp
Total number of bands		**2 (lost)**	**7 (lost)**	**8 (lost); 1 (new)**	**5 (lost); 2 (new)**
CDDP					
TaWRKY1F	+	-	500 bp	-	500
	-	-	600 bp	-	-
TaWRKY1R	+	800 bp	800 bp	-	-
	-	-	1550; 700 bp	3500; 2700; 2600; 2400; 2100; 1550; 1500; 700 bp	3500; 2700; 2600; 2400; 2050; 1550; 1500; 900; 700; 300 bp
TaWRKY33R	+	-	-	-	-
	-	3000; 1750; 1100; 1000 bp	3000; 2500; 2250; 1750; 1100; 1000 bp	2250; 2000; 1500; 1000 bp	2250; 2000; 1500; 1100; 1000; 800 bp
TaWRKY44F	+	-	-	550	550 bp
	-	-	-	2750; 2500; 510 bp	2750; 2000; 510 bp
TaWRKY44R	+	3100; 2700; 2300 bp	2300 bp	2900; 2700 bp	2900 bp
	-	300 bp	-	2400; 300 bp	2400; 300 bp
MQC-F	+	2400 bp	-	3100; 2200 bp	3100; 2400; 2200 bp
	-	2000; 900; 650 bp	2000; 900; 650 bp	-	3000; 1700; 1500; 1200; 1000; 450 bp
MQC-R	+	-	1400; 800 bp	-	-
	-	1000; 900 bp	1000; 900 bp	2500; 1400; 1000 bp	2500; 1000; 900 bp
Total number of bands		**10 (lost); 5 (new)**	**11 (lost); 5 (new)**	**20 (lost); 5 (new)**	**30 (lost); 6 (new)**

In plants treated with AsA, the molecular size of polymorphic SCoT bands (new and lost) ranged from 500 bp to 2,550 bp (Table 5).

The molecular size of polymorphic CDDP bands detected in plants treated with AsA ranged from 300 to 3,500 bp (Table 5).

Regarding the sum of disappeared bands, the values were higher in the induced drought regime, particularly for CDDP markers, and in the treatment AsA priming plus foliar application (Table 5). The highest number of lost SCoT bands was registered in the drought regime in plants treated with AsA priming (Table 5).

For both markers, the number of new bands was higher in the drought regime (Table 5).

Considering that the percentage of polymorphism resultant from the comparison among the molecular profiles observed in the control and treated plants may be indicative of genomic instability or remodelling caused by the abiotic stress, in Table 6 it is presented the total percentage of SCoT and CDDP polymorphism achieved per AsA-treatment in both water regimes.

Table 6. Percentage of SCoT and CDDP polymorphism determined per AsA-treatment in both water regimes.

Marker	**Percentage of polymorphism (%P) in:**			
	Watering		**Drought**	
	AsA priming	**AsA priming and foliar application**	**AsA priming**	**AsA priming and foliar application**
SCoTs	16.7%	39.6%	41.0%	42.0%
CDDPs	33.1%	45.4%	42%	57.7%

The SCoT markers revealed less genomic instability in plants treated with AsA priming in both watering (16.7%) and drought (41%) regimes (Table 6). Similarly, the lowest values of polymorphism or genomic instability assessed by CDDP markers were found in plants treated with AsA priming in both watering (33.1%) and drought (42%) regimes (Table 6).

3.2.5.2. Zn-treated and control plants characterized by gene-targeted markers

Table 7 summarizes the SCoT and CDDP data resultant of the comparison of molecular patterns among Zn-treated and control plants of both water regimes.

Table 7. Molecular size (bp) and number of newly appeared (+) and disappeared (-) SCoT and CDDP bands relative to control plants detected per primer in Zn-treated plants of both water regimes.

Marker/ Primer		Watering		Drought	
		Zn priming	Zn priming and foliar application	Zn priming	Zn priming and foliar application
SCoT					
SCoT 1	+	-	-	-	-
	-	500 bp	-	1100 bp	-
SCoT 2	+	-	-	-	-
	-	950 bp	-	2300; 1700; 1100; 600 bp	600 bp
SCoT 4	+	-	-	-	-
	-	-	-	2550; 2100; 1300 bp	2550; 2100; 1300; 700 bp
SCoT 7	+	-	-	2300 bp	2300 bp
	-	-	1400 bp	2000; 1600; 450 bp	-
SCoT 11	+	-	-	-	-
	-	1500; 1000; 900; 850; 750 bp	-	500 bp	2250; 500 bp
Total number of bands		**7 (lost)**	**1 (lost)**	**12 (lost); 1 (new)**	**7 (lost); 1 (new)**
CDDP					
TaWRKY1F	+	-	-	-	2600 bp
	-	-	600 bp	-	-
TaWRKY1R	+	-	800 bp	-	-
	-	1550 bp	1700; 1550; 1500; 900 bp	3500; 2600; 2400; 700 bp	2700; 2100; 700 bp
TaWRKY33R	+	-	-	-	-
	-	-	3000; 1100; 1000 bp	2000; 1500 bp	2250; 2000; 1500 bp
TaWRKY44F	+	-	-	-	550 bp
	-	-	1250 bp	2750; 510 bp	2750; 2500; 2000; 510; 400 bp
TaWRKY44R	+	3100; 2900; 2700; 2300 bp	2300 bp	2900; 2700 bp	-
	-	-	300 bp	2400; 1500; 300 bp	2400; 1500; 300 bp
MQC-F	+	-	-	2200 bp	3100 bp
	-	2700; 1600; 550 bp	900; 650 bp	-	2000; 1700; 1500; 1200; 450 bp
MQC-R	+	800 bp	1400; 800 bp	-	-
	-	1000 bp	1000; 900; 600 bp	1400 bp	2500; 1400; 1000; 900 bp
Total number of bands		**5 (lost); 5 (new)**	**15 (lost); 4 (new)**	**12 (lost); 3 (new)**	**23 (lost); 3 (new)**

The number of lost SCoT bands was higher in plants under drought particularly in those treated with Zn priming (Table 7).

In the case of CDDP markers, the higher number of lost bands was also registered in plants under drought namely in those treated with Zn priming plus foliar application (Table

7). On the other hand, a higher number of new CDDP bands was detected in watered plants (Table 7).

The Zn-treated plants showed polymorphic SCoT bands ranging in size from 500 bp to 2,550 bp, and polymorphic CDDP bands varying in size from 300 to 3,500 bp (Table 7).

Some lost CDDP bands were common in plants treated with Zn priming and treated with Zn priming plus foliar application of the drought regime (Table 7).

Table 8 presents the total percentage of SCoT and CDDP polymorphism achieved per Zn-treatment in both water regimes.

Table 8. Percentage of SCoT and CDDP polymorphism determined per Zn-treatment in both water regimes.

Marker	Percentage of polymorphism (%P) in:			
	Watering		Drought	
	Zn priming	Zn priming and foliar application	Zn priming	Zn priming and foliar application
SCoTs	30.8%	4.0%	73.0%	33.0%
CDDPs	27.7%	33%	27.9%	50%

The SCoT markers revealed lower percentages of polymorphism in plants treated with Zn priming plus foliar application in both watering (4.0%) and drought (33.0%) regimes (Table 8). Inversely, the CDDP markers showed lower percentages of polymorphisms in plants treated with Zm priming in both watering (27.7%) and drought (27.9%) regimes (Table 8).

3.2.6. Polymorphism among treated and control plants evidenced by RTNs-based and ISSR markers

On a first approach, single LTR, SSR and PBS primers were tested individually. Based on the successful amplification achieved with those single primer reactions, combinations of primers were tested accordingly previous studies performed in wheat and other cereals, and some of them were selected (see Table 3) for the amplification of IRAP, REMAP, ISSR and/or iPBS markers in this work (Tables 9 and 11).

Overall, for the amplification of IRAP and REMAP markers, five and three combinations of primers, respectively, were selected, whereas the iPBS and ISSR markers

were produced by a single primer, the PBS primer F0100 and the SSR primer 8081, respectively (Tables 9 and 11).

Figure 15 shows REMAP polymorphic patterns produced by the combination of primers LTR 7286 + SSR 8081 among control, AsA- and Zn-treated plants of each water availability regime.

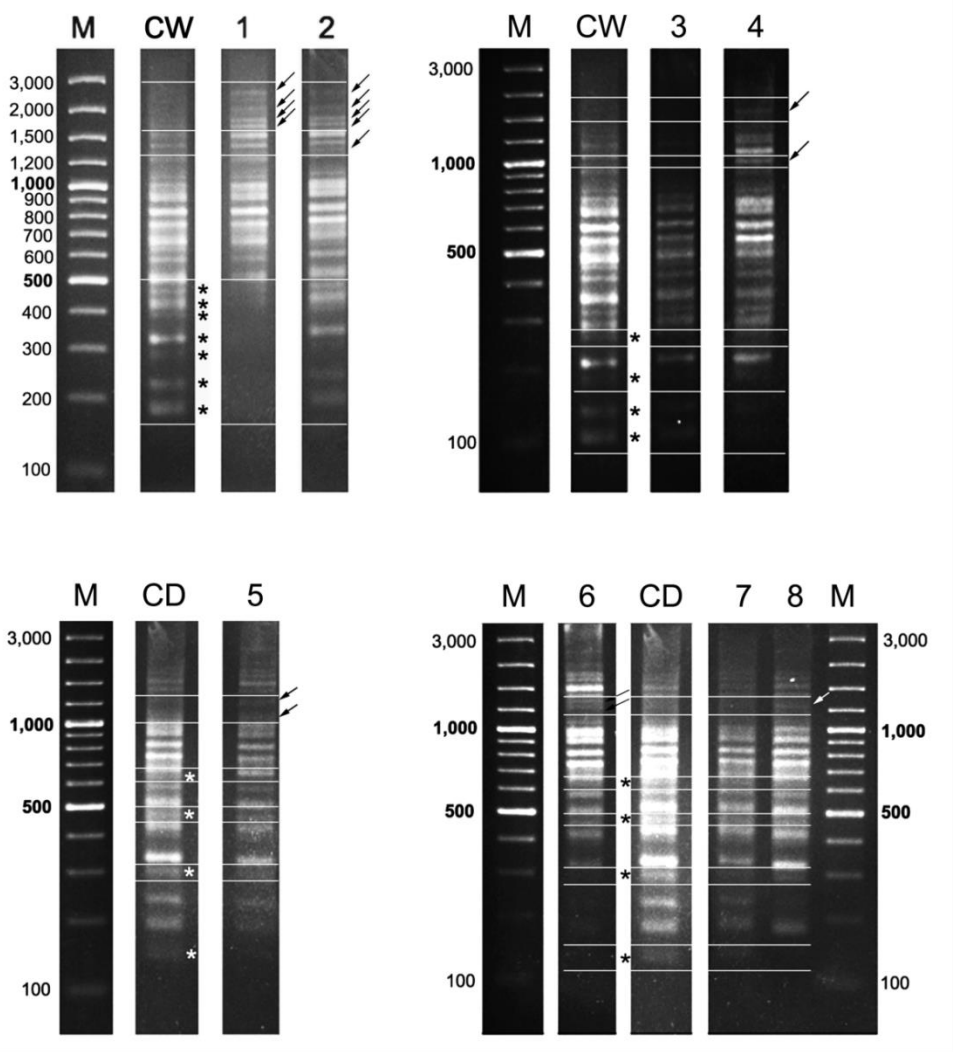


Figure 15. REMAP polymorphic patterns produced by the primers LTR 7286 + SSR 8081 in plants under: watering (*upper scheme*) and drought (*lower scheme*) regimes. Note: CW - watering control; lane 1 - plant treated with Zn priming; lane 2 – plant treated with Zn priming + foliar spraying; lane 3 – plant treated with AsA priming; lane 4 – plant treated with AsA priming + foliar spraying; CD - control in drought; lane 5 - plant treated with Zn priming; lane 6 – plant treated with Zn priming + foliar spraying; lane 7 – plant treated with AsA priming; lane 8 – plant treated with AsA priming + foliar spraying. Arrows indicate new bands and asterisk (*) indicates in the control plants those bands that disappeared in treated plants of the same water regime. M – Molecular weight marker.

Figure 15 is representative of the remaining gels resultant of the amplification of others RTNs-based markers and ISSRs in AsA- and Zn-treated plants of both water regimes and respective controls.

3.2.6.1. AsA-treated and control plants characterized by RTNs-based and ISSR markers

Table 9 presents the IRAP, REMAP, iPBS and ISSR data in plants treated with AsA after comparison with the molecular patterns of the control plants in each water availability regime.

Table 9. Molecular size (bp) and number of newly appeared (+) and disappeared (-) IRAP, REMAP, iPBS and ISSR bands relative to control plants detected per primer in AsA-treated plants of both water regimes.

Marker/ Primer(s)		Watering		Drought	
		AsA priming	**AsA priming and foliar application**	**AsA priming**	**AsA priming and foliar application**
IRAP					
5'LTR + *Sukkula*	+	-	-	600 bp	600 bp
	-	550; 500 bp	900; 800; 550; 500 bp	-	-
6149 + *Sukkula*	+	950 bp	950 bp	-	950 bp
	-	1300; 650 bp	1500; 1300; 1100; 1000; 950; 800; 700; 650; 550 bp	1300 bp	2300; 2100; 1300; 900; 550; 500 bp
6150 + *Sukkula*	+	2500 bp	-	-	-
	-	1400; 1200, 850; 700; 600 bp	1400; 850; 800; 700; 600 bp	-	-
Nikita + *Sukkula*	+	-	-	2500 bp	-
	-	1400; 950 bp	1400; 950 bp	1500; 1100 bp	2400; 1500; 1400; 1100; 950; 900; 600 bp
Sukkula	+	-	-	-	-
	-	1900; 1400; 750 bp	2400; 2200; 1900; 1400; 850; 800; 750 bp	1900; 350 bp	2400; 1900; 1100; 850; 800; 650 bp
Total number of bands		**14 (lost); 2 (new)**	**27 (lost); 1 (new)**	**5 (lost); 2 (new)**	**19 (lost); 2 (new)**
REMAP					
7286 + 8081	+	-	1,600, 1,000 bp	-	1,300 bp
	-	380, 180 bp	150, 100 bp	450; 280 bp	650; 150 bp
Nikita + 8081	+	-	-	600 bp	600 bp
	-	1700; 450; 400 bp	1700; 450; 400 bp	2300; 950 bp	2700; 2300; 950; 850 bp
Sukkula + 8081	+	950 bp	950 bp	-	-
	-	1200; 450; 400 bp	1200; 800; 400 bp	600 bp	1200; 800 bp
Total number of bands		**8 (lost); 1 (new)**	**8 (lost); 3 (new)**	**5 (lost); 1 (new)**	**8 (lost); 2 (new)**
iPBS					
F0100	+	-	-	-	-
	-	-	-	2600; 1500; 150 bp	2600; 2400; 2300; 1800; 1500 bp
Total number of bands		**-**	**-**	**3 (lost)**	**5 (lost)**
ISSR					
8081	+	-	-	2500 bp	1000 bp
	-	2200 bp	-	-	-
Total number of bands		**1 (lost)**	**-**	**1 (new)**	**1 (new)**

The highest number of lost bands in AsA-treated plants was detected with the IRAP markers, in both water regimes, particularly, in those treated with AsA priming plus foliar application (Table 9).

Regarding both water regimes most of the treatments showed the loss of eight REMAP bands (Table 9).

A few number of lost and/or new iPBS and ISSR bands was detected (Table 9). However, the higher number of lots iPBS bands (eight) was registered in plants under drought (Table 9).

Since the percentage of polymorphism may be indicative of genomic instability, the total percentage values of IRAP, REMAP, ISSR and iPBS polymorphism achieved per AsA-treatment in both water regimes are presented in Table 10.

Table 10. Percentage of IRAP, REMAP, iPBS and ISSR polymorphism determined per AsA-treatment in both water regimes.

Marker	Percentage of polymorphism (%P) in:			
	Watering		Drought	
	AsA priming	AsA priming and foliar application	AsA priming	AsA priming and foliar application
IRAP	47.52%	68.18%	12.24%	35.58%
REMAP	47.33%	55.67%	31%	53.33%
iPBS	0%	0%	22.2%	33.3%
ISSR	10%	0%	10%	10%

The IRAP and REMAP markers revealed lowest polymorphism percentages in plants treated with AsA priming in both water regimes (Table 10).

The iPBS polymorphism was only detected in plants submitted to drought, and that value was lower in plants treated with AsA priming (Table 10).

Ten percent of ISSR polymorphism was observed in all treatments of both water regimes except for watered plants treated with AsA priming plus foliar spraying (Table 10).

3.2.6.2. Zn-treated and control plants characterized by RTNs-based and ISSR markers

Table 11 summarizes the IRAP, REMAP, iPBS and ISSR data, achieved in the Zn-treated plants of both water regimes.

Table 11. Molecular size (bp) and number of newly appeared (+) and disappeared (-) IRAP, REMAP, iPBS and ISSR bands relative to control plants detected per primer in Zn-treated plants of both water regimes.

Marker/ Primer(s)		Watering		Drought	
		Zn priming	**Zn priming and foliar application**	**Zn priming**	**Zn priming and foliar application**
IRAP					
5'LTR + *Sukkula*	+	-	-	600 bp	600 bp
	-	1100; 550 bp	1000; 900; 550 bp	2400; 550 bp	1400 bp
6149 + *Sukkula*	+	2700; 950 bp	2700; 950 bp	-	-
	-	1500; 1300; 1100; 1000; 900; 800; 700; 650; 550 bp	1300; 900; 650 bp	1300 bp	1300; 500 bp
6150 + *Sukkula*	+	2500 bp	2500bp	-	-
	-	1400; 850; 800; 700; 600 bp	1500; 1400; 1200; 1000; 850; 800; 700; 600 bp	-	-
Nikita + *Sukkula*	+	-	-	2500 bp	2500 bp
	-	-	1400; 950 bp	1500; 1100; 950 bp	600 bp
Sukkula	+	400 bp	-	-	-
	-	2400; 2200; 1900; 1600; 1400; 1100; 1000; 850; 800; 750; 700; 650; 550; 500 bp	2400; 2200; 1900; 1400; 1000; 850; 800; 750; 700; 650; 550; 500 bp	-	2400; 200; 1900; 1400; 850; 800; 750 bp
Total number of bands		**30 (lost); 4 (new)**	**28 (lost); 3 (new)**	**6 (lost); 2 (new)**	**11 (lost); 2 (new)**
REMAP					
7286 +8081	+	2500, 2000, 1800 bp	2500, 2000, 1800, 1300, 1100 bp	1200, 1100 bp	1300, 1200 bp
	-	480, 425, 400, 325, 280, 225, 180 bp	280 bp	650, 150 bp	280, 150 bp
Nikita + 8081	+	-	-	600 bp	600 bp
	-	-	1700; 950; 450; 400 bp	2300 bp	2300; 950 bp
Sukkula + 8081	+	950 bp	950 bp	-	-
	-	1200; 800; 450; 400 bp	2100; 1200; 800; 450; 400 bp	-	-
Total number of bands		**11 (lost); 4 (new)**	**10 (lost); 6 (new)**	**3 (lost); 3 (new)**	**4 (lost); 3 (new)**
iPBS					
F0100	+	-	-	-	-
	-	650 bp	1200; 650 bp	-	2600; 1500 bp
Total number of bands		**1 (lost)**	**2 (lost)**	**-**	**2 (lost)**
ISSR					
8081	+	1000 bp	1000bp	-	-
	-	-	2600; 1800; 550 bp	-	-
Total number of bands		**1 (new)**	**3 (lost); 1 (new)**	**-**	**-**

The IRAP markers showed the highest number of lost bands in both Zn-treatments of the watering regime (Table 11). In plants under drought, the highest number of lost IRAP bands was detected in those treated with Zn priming plus foliar application (Table 11).

The highest number of lost REMAP bands was also registered in watered plants (Table 11). Both markers, IRAP and REMAP also revealed the highest number of new bands in watered plants (Table 11).

The iPBS markers only detected a few number of lost bands and none polymorphic band in plants under drought treated with Zn priming (Table 11).

The highest number of polymorphic ISSR bands were noticed in watered plants treated with Zn priming + foliar application (Table 11).

The total percentages of polymorphism achieved with IRAP, REMAP, ISSR and iPBS markers in Zn-treated plants relative to the controls of both water regimes are presented in Table 12.

Table 12. Percentage of IRAP, REMAP, ISSR and iPBS polymorphism determined per Zn-treatment in both water regimes

Marker	Percentage of polymorphism (%P) in:			
	Watering		Drought	
	Zn priming	Zn priming and foliar application	Zn priming	Zn priming and foliar application
IRAP	57.78%	72.90%	15.94%	22.86%
REMAP	57%	80.33%	23%	28.67%
iPBS	100%	100%	0%	22.22%
ISSR	0%	20%	0%	0%

Both IRAP and REMAP markers showed lower percentages of polymorphism in plants treated with Zn priming in both water regimes (Table 12).

The iPBS markers revealed 100% of polymorphism in all watered plants (Table 12). In plants under drought, we only found iPBS polymorphism in plants treated with Zn priming + foliar application (Table 12).

ISSR polymorphism was only detected in watered plants treated with Zn priming + foliar application (Table 12).

3.2.7. Polymorphism among treated and control plants evidenced by RAPD markers

Among the 13 RAPD tested primers, seven showed successful amplification as well as polymorphic patterns among the control, AsA- and Zn-treated plants of each water regime (Tables 13 and 15). Figure 16 shows polymorphic RAPD patterns produced by primer OPB13 among control, AsA- and Zn-treated plants of both water regimes.

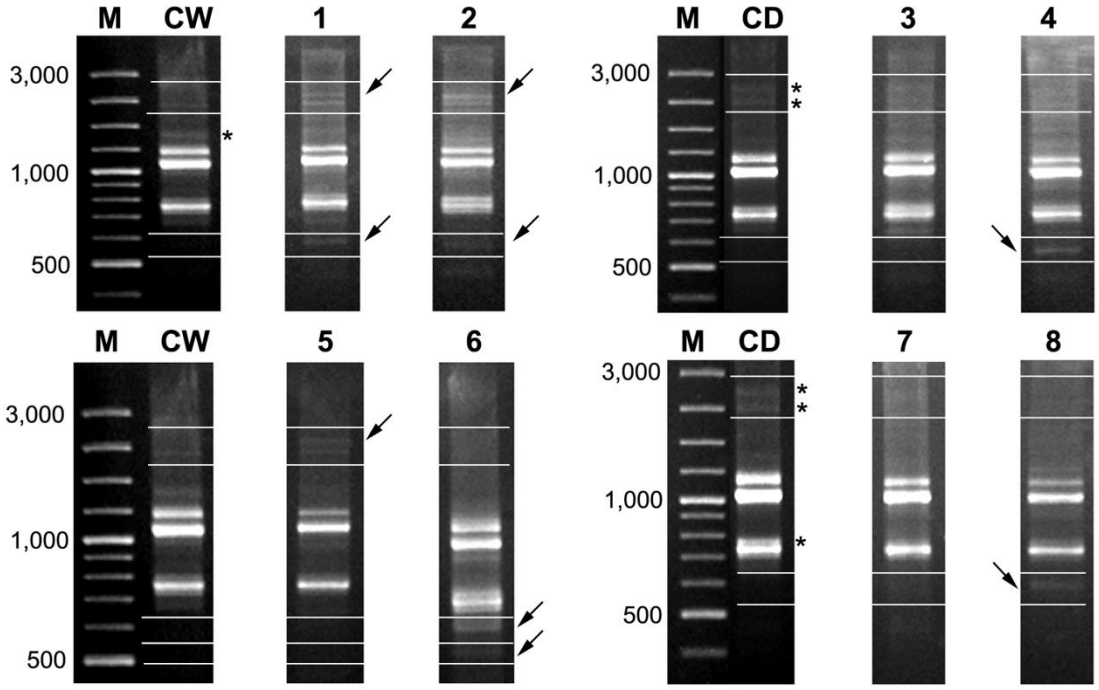


Figure 16. RAPD polymorphic patterns produced with primer OPB13 among: the watering control (CW) and plants treated with Zn priming (lane 1); Zn priming plus foliar spraying (lane 2); AsA priming (lane 5) and AsA priming plus foliar spraying (lane 6); and among the drought control (CD) and plants treated with Zn priming plus foliar application (lane 4); AsA priming (lane 7) and AsA priming plus foliar spraying (lane 8). One plant under drought treated with Zn priming (lane 3) showed a similar molecular pattern to its respective control (CD). Arrows indicate new bands whereas the asterisk (*) indicates in the control plants those bands that disappeared in treated plants of the same water regime. M – Molecular weight marker.

The gels presented in Fig. 16 are representative of the remaining ones produced by additional RAPD primers in all treatments of both water regimes.

3.2.7.1. AsA-treated and control plants characterized by RAPD markers

Table 13 summarizes the newly appeared and disappeared RAPD bands in AsA-treated plants of both water availability regimes.

Table 13. Molecular size (bp) and number of newly appeared (+) and disappeared (-) RAPD bands relative to control plants detected per primer in AsA-treated plants of both water regimes.

Primer		Watering		Drought	
		AsA priming	**AsA priming and foliar application**	**AsA priming**	**AsA priming and foliar application**
OPB01	+	-	-	-	-
	-	500 bp	900 bp	1900; 700; 300 bp	-
OPB02	+	-	-	-	-
	-	400 bp	-	2400; 2000; 1600; 1000; 800; 600; 550 bp	2400; 2000; 1600; 550bp
OPB05	+	3000; 2600; 1000; 700 bp	3000; 2400; 1000; 700; 600; 500 bp	700 bp	700 bp
	-	-	-	2000 bp	2000 bp
OPB06	+	-	-	-	-
	-	2100; 1400; 1000 bp	2100; 1600; 1400; 1000; 900 bp	1600; 1400; 900 bp	1600 bp
OPB08	+	-	-	-	-
	-	900; 750 bp	750; 550 bp	-	900; 850 bp
OPB12	+	-	-	-	-
	-	-	2600; 1900; 1500 bp	390 bp	-
OPB13	+	2600 bp	600; 500 bp	-	600 bp
	-	-	-	2750; 2500; 750 bp	2750; 2500; 750 bp
Total number of bands		**7 (lost); 5 (new)**	**11 (lost); 8 (new)**	**18 (lost); 1 (new)**	**11 (lost); 2 (new)**

The higher number of lost RAPD bands was detected in plants under drought treated with AsA priming (Table 13). However, if we consider all polymorphic bands, it was detected 31 in the watering regime and 32 in drought (Table 13). The higher number of new bands was detected in the watered plants (Table 13).

In plants treated with AsA, the molecular size of polymorphic RAPD bands ranged from 300 bp to 3,000 bp (Table 13). A unique new band with 2,600 bp, amplified with the primer OPB13, appeared only in watered plants treated with AsA priming. On the other hand,

a 600 bp band occured in plants under drought treated with AsA priming plus foliar application (Table 13).

'Normal bands' with 2,000 bp (amplified with primer OPB05), 2,750 bp, 2,500 bp and 750 bp (amplified with primer OPB13) in the drought control (CD) disappeared in plants under drought and treated with both AsA priming, and AsA priming + foliar spraying (Table 13).

Concerning to the percentages of RAPD polymorphism achieved in all AsA treatments and in both water regimes, the lowest value (27.1%) was achieved in plants under drought and treated with AsA priming plus foliar spraying (Table 14).

Table 14. Percentage of RAPD polymorphism determined per AsA-treatment in both water regimes

Primer	Percentage of RAPD polymorphism (%P) in:			
	Watering		Drought	
	AsA priming	AsA priming and foliar spraying	AsA priming	AsA priming and foliar spraying
OPB01	16.7%	16.7%	37.5%	0%
OPB02	100%	0%	87.5%	50%
OPB05	50%	75%	66.7%	66.7%
OPB06	50%	83.3%	75%	25%
OPB08	22.2%	22.2%	0%	20%
OPB12	0%	21.4%	6.25%	0%
OPB13	0%	20%	28.6%	28.6%
Average	**34.1%**	**34.1%**	**43%**	**27.1%**

The watered plants showed the same percentage of polymorphism (34.1%) in both AsA priming and AsA priming plus foliar spraying (Table 14).

3.2.7.2. Zn-treated and control plants characterized by RAPD markers

Table 15 summarizes the RAPD data achieved in plants treated with Zn.

Table 15. Molecular size (bp) and number of newly appeared (+) and disappeared (-) RAPD bands relative to control plants detected per primer in Zn-treated plants of both water regimes

Primer		Watering		Drought	
		Zn priming	**Zn priming and foliar spraying**	**Zn priming**	**Zn priming and foliar spraying**
OPB01	+	-	-	-	-
	-	-	-	-	1900 bp
OPB02	+	-	-	-	-
	-	-	400 bp	-	2400; 2000; 1600; 1000; 800; 600; 550 bp
OPB05	+	1000 bp	2400; 1000 bp	700 bp	700 bp
	-	-	-	2000 bp	2000 bp
OPB06	+	-	-	-	-
	-	-	2100; 1600; 1400 bp	-	-
OPB08	+	-	-	-	-
	-	-	750 bp	1900 bp	1900; 650 bp
OPB12	+	-	-	-	-
	-	490 bp	2600; 1900; 1500; 1300; 900; 850 bp	-	-
OPB13	+	2600; 600 bp	2600; 600 bp	-	600 bp
	-	-	-	-	2750; 2500 bp
Total number of bands		**1 (lost); 3 (new)**	**10 (lost); 4 (new)**	**2 (lost); 1 (new)**	**13 (lost); 2 (new)**

Concerning the sum of lost RAPD bands in each water regime, the number was higher in drought, particularly, in plants treated with Zn priming plus foliar spraying (Table 15).

The molecular size of the polymorphic RAPD bands detected in the Zn-treated plants ranged from 400 bp to 2,400 bp (Table 15).

Three RAPD bands (2,100 bp, 1,600bp and 1,400 bp) were amplified in control plants with the primer OPB06 but they were absent in watered plants treated with both Zn treatments. The same happened with the band of 400 bp amplified with the primer OPB02 and the band of 1,900 bp amplified with the primer OPB01 (Table 15).

In all Zn-treated plants under drought, a new RAPD band with 700 bp, amplified with the primer OPB05, was detected (Table 15).

Table 16 presents the percentage of RAPD polymorphism achieved per primer in all Zn treatments of both water regimes.

Table 16. Percentage of RAPD polymorphism determined per Zn-treatment in both water regimes

Primer	Percentage of RAPD polymorphism (%P) in:			
	Watering		Drought	
	Zn priming	Zn priming and foliar spraying	Zn priming	Zn priming and foliar spraying
OPB01	0%	0%	0%	12.5%
OPB02	0%	100%	0%	87.5%
OPB05	12.5%	25%	66.7%	66.7%
OPB06	0%	50%	0%	0%
OPB08	0%	11.1%	10%	20%
OPB12	7.1%	42.9%	0%	0%
OPB13	20%	20%	0%	14.3%
Average	**5.7%**	**35.6%**	**11%**	**28.7%**

Among the Zn treatments, Zn-priming evidenced the lowest values of RAPD polymorphism in both water regimes (Table 16). Plants under drought treated with Zn priming plus foliar application presented lower RAPD polymorphism than those watered and treated with the same way (Table 16).

3.2.8. Statistical analyses of the molecular data

Regarding that the genomic instability is revealed by the polymorphism, the statistical analyses were based on the sum of lost and new bands achieved per marker system, treatment and/or water regime, aiming the determination of which of these variable(s) or effect(s) influenced more the scored number of new and lost bands.

3.2.8.1. SCoT and CDDP markers

The statistical results of the SCoT and CDDP data are summarized in Table 17.

Table 17. Mean values ± S.E. of new and lost bands determined per water availability regime, treatment, marker system (CDDP and SCoT), and respective interactions among these variables. Mean values within a column followed by different lower case letters represent statistically significant differences for a significance level of 95% ($p < 0.05$) determined with the Equality of variances F test and Fisher's PLSD test.

Variables or effects		**New bands (Mean ± S.E.)**	**Lost bands (Mean ± S.E.)**
Water regime (W)	Watering	2.375 ± 0.905	7.250 ± 1.656
	Drought	2.750 ± 0.675	14.625 ± 3.116
Treatment (T)	AsA priming	2.75 ± 1.315	10.00 ± 3.742
	AsA priming and foliar	3.25 ±1.377	13.25 ± 5.721
	Zn priming	2.25 ± 1.109	9.00 ± 1.78
	Zn priming and foliar	2.00 ±0.913	11.50 ± 4.787
Marker (M)	CDDP	4.50±0.378 a	15.75±2.864 a
	SCoT	0.625±0.263 b	6.125±1.231 b
W x T	Watering x AsA priming	2.50±2.50	6.00±4.00
	Watering x AsA priming and foliar	2.50±2.50	9.00±2.00
	Watering x Zn priming	2.50±2.50	6.00±1.00
	Watering x Zn priming and foliar	2.00±2.00	8.00±7.00
	Drought x AsA priming	3.00±2.00	14.00±6.00
	Drought x AsA priming and foliar	4.00±2.00	17.50±12.50
	Drought x Zn priming	2.00±1.00	12.00±0.00
	Drought x Zn priming and foliar	2.00±1.00	15.00±8.00
W x M	Watering x CDDP	4.75±0.25	10.25±2.056
	Watering x SCoT	0.00±0.00	4.25±1.601
	Drought x CDDP	4.25±0.75	21.25±3.728
	Drought x SCoT	1.25±0.25	8.00±1.472
T x M	AsA priming x CDDP	5.00±0.00	15.00±5.00
	AsA priming x SCoT	0.50±0.50	5.00±3.00
	AsA priming and foliar x CDDP	5.50±0.50	20.50±9.50
	AsA priming and foliar x SCoT	1.00±1.00	6.00±1.00
	Zn priming x CDDP	4.00±1.00	8.50±3.50
	Zn priming x SCoT	0.50±0.50	9.50±2.50
	Zn priming and foliar x CDDP	3.50±0.50	19.00±4.00
	Zn priming and foliar x SCoT	0.50±0.50	4.00±3.00
***p* - values**			
W		$p > 0.05$	$p < 0.05$
T		$p > 0.05$	$p > 0.05$
M		$p < 0.0001$	$p < 0.05$
W xT		$p > 0.05$	$p > 0.05$
W x M		$p > 0.05$	$p > 0.05$
T x M		$p > 0.05$	$p > 0.05$

The number of lost bands statistically differed ($p < 0.05$) between the two water regimes (W) (Table 17). Both the number of new ($p < 0.0001$) and lost ($p < 0.05$) bands statistically differed between the markers (M), SCoTs and CDDPs (Table 17).

No statistically significant differences ($p > 0.05$) were found for new and lost bands among the four treatments (T) neither for the interactions W x T, W x M or T x M (Table 17).

3.2.8.2. RTNs-based and ISSR markers

In Table 18 are summarized the statistical results of the molecular data produced with the RTNs-based markers and ISSRs.

Table 18. Mean values ± S.E. of new and lost bands determined per water availability regime, treatment, marker system (RTNs-based), and respective interactions among these variables. Mean values within a column followed by different lower case letters represent statistically significant differences for a significance level of 95% ($p < 0.05$) determined with the Equality variances F test and Fisher's PLSD test. Note: n.d. - means no determined.

Variables or effects		**New bands (Mean ± S.E.)**	**Lost bands (Mean ± S.E.)**
Water regime (W)	Watering	0.81 ± 0.26	9.00 ± 0.26 a
	Drought	0.75 ± 0.19	4.25 ± 1.30 b
Treatment (T)	AsA priming	0.88 ± 0.30	4.88 ± 1.89
	AsA priming and foliar	0.50 ± 0.19	9.13 ± 3.52
	Zn priming	0.88 ± 0.40	5.38 ± 3.62
	Zn priming and foliar	0.88 ± 0.40	7.13 ± 3.41
Marker (M)	IRAP	2.00 ± 0.27 e	16.63 ± 3.91 e
	REMAP	1.00 ± 0.00 d	8.13 ± 1.56 d
	iPBS	n.d.	1.38 ± 0.60 c
	ISSR	0.13 ± 0.13 c	0.38 ± 0.26 c
W x T	Watering x AsA priming	0.75 ± 0.48	6.25 ± 3.54
	Watering x AsA priming and foliar	0.50 ± 0.29	9.25 ± 6.37
	Watering x Zn priming	1.00 ± 0.71	9.00 ± 7.08
	Watering x Zn priming and foliar	1.00 ± 0.71	11.50 ± 6.36
	Drought x AsA priming	1.00 ± 0.41	3.50 ± 1.71
	Drought x AsA priming and foliar	0.50 ± 0.29	9.00 ± 4.14
	Drought x Zn priming	0.75 ± 0.48	1.75 ± 1.44
	Drought x Zn priming and foliar	0.75 ± 0.48	2.75 ± 1.11
W x M	Watering x IRAP	2.25 ± 0.48	25.00 ± 3.39
	Watering x REMAP	1.00 ± 0.00	9.75 ± 2.06
	Watering x iPBS	n.d.	0.50 ± 0.29
	Watering x ISSR	n.d.	0.75 ± 0.48
	Drought x IRAP	1.75 ± 0.25	8.25 ± 3.61
	Drought x REMAP	1.00 ± 0.00	6.50 ± 2.33
	Drought x iPBS	n.d.	2.25 ± 1.03
	Drought x ISSR	0.25 ± 0.25	n.d.
T x M	AsA priming x IRAP	n.d.	9.50 ± 5.50
	AsA priming x REMAP	1.00 ± 0.00	8.50 ± 0.50
	AsA priming x iPBS	n.d.	1.00 ± 1.00
	AsA priming x ISSR	0.50 ± 0.50	0.50 ± 0.50
	AsA priming and foliar x IRAP	1.00 ± 0.00	2.30 ± 4.00
	AsA priming and foliar x REMAP	1.00 ± 0.00	11.00 ± 1.00
	AsA priming and foliar x iPBS	n.d.	2.50 ± 2.50
	AsA priming and foliar x ISSR	n.d.	n.d.
	Zn priming x IRAP	2.50 ± 0.00	18.00 ± 12.00
	Zn priming x REMAP	1.00 ± 0.00	3.00 ± 2.00
	Zn priming x iPBS	n.d.	0.50 ± 0.50
	Zn priming x ISSR	n.d.	n.d.
	Zn priming and foliar x IRAP	2.50 ± 0.50	16.00 ± 12.00
	Zn priming and foliar x REMAP	1.00 ± 0.00	10.00 ± 5.00
	Zn priming and foliar x iPBS	n.d.	1.50 ± 0.50
	Zn priming and foliar x ISSR	n.d.	1.00 ± 1.00
***p* - values**			
W		$p > 0.05$	$p < 0.05$
T		$p > 0.05$	$p > 0.05$
M		$p < 0.0001$	$p < 0.0001$
W xT		$p > 0.05$	$p > 0.05$
W x M		$p > 0.05$	$p < 0.05$
T x M		$p < 0.05$	$p > 0.05$

The number of lost bands statistically differed ($p < 0.05$) between the two water regimes (Table 18). Both the number of new and lost bands statistically differed ($p < 0.001$) among the various marker systems used (Table 18) except between the markers ISSR and iPBS ($p > 0.05$) (Table 18). However, regarding the interactions water regime (W) x marker system (M) and treatment (T) x marker system (M), statistically significant differences ($p < 0.05$) were found for the number of lost and new bands, respectively (Table 18).

No statistically significant differences ($p > 0.05$) were found for both new and lost bands among the four treatments (Table 18).

According to the Fisher's PLSD test and regarding the effect treatment, in the case of the new bands amplified with the RTNs-based markers and ISSRs it were found highly statistically significant differences ($p < 0.0001$) between the following pairs of markers systems: iPBS and IRAP, iPBS and REMAP, IRAP and ISSR, IRAP and REMAP, as well as between ISSR and REMAP ($p = 0.0002$).

For the lost bands and effect treatment, the Fisher's PLSD test revealed highly statistically significant differences ($p < 0.0001$) between the following pairs of marker systems: iPBS and IRAP, IRAP and ISSR, as well as between iPBS and REMAP, ISSR and REMAP, IRAP and REMAP ($p < 0.05$). No statistically significant differences ($p > 0.05$) were found between iPBS and ISSRs for both new and lost bands regarding the effect treatment.

Concerning to the effect marker system, for the number of lost bands were found highly statistically significant differences ($p < 0.0001$) between iPBS and IRAP, IRAP and ISSR. Statistically significant differences ($p < 0.05$) for the lost bands were also found between iPBS and REMAP, ISSR and REMAP, IRAP and REMAP. In the case of new bands, highly statistically significant differences ($p < 0.0001$) between iPBS and IRAP, iPBS and REMAP, IRAP and ISSR, IRAP and REMAP, as well as between ISSR and REMAP ($p < 0.05$) were found.

No statistically significant differences ($p > 0.05$) were found between iPBS and ISSRs for both new and lost bands regarding the effect marker.

3.2.8.3. RAPD markers

The statistical analyses were performed based on the sum of lost and new RAPD bands produced in both watering regimes and in all treatments. The statistical results are summarized in Table 19.

Table 19. Mean values ± S.E. of new and lost bands determined per water availability regime, treatment and their interaction after RAPD amplification. Mean values within a column followed by different lower case letters represent statistically significant differences for a significance level of 95% ($p < 0.05$) determined with the Equality variances F test and Fisher's PLSD test.

Variables or effects		**New bands (Mean ± S.E.)**	**Lost bands (Mean ± S.E.)**
Water regime (W)	Watering	4.00 ± 1.08 a	7.25 ± 2.25
	Drought	1.25 ± 0.25 b	9.50 ± 2.90
Treatment (T)	AsA priming	2.50 ± 1.50	11.50 ± 4.50 b
	AsA priming and foliar	4.00 ± 3.00	10.00 ± 1.00 b
	Zn priming	1.50 ± 0.50	1.50 ± 0.50 a
	Zn priming and foliar	2.50 ± 0.50	10.50 ± 0.50 b
W x T	Watering x AsA priming	4.00 ± 0.00	7.00 ± 0.00
	Watering x AsA priming and foliar	7.00 ± 0.00	11.00 ± 0.00
	Watering x Zn priming	2.00 ± 0.00	1.00 ± 0.00
	Watering x Zn priming and foliar	3.00 ± 0.00	10.00 ± 0.00
	Drought x AsA priming	1.00 ± 0.00	16.00 ± 0.00
	Drought x AsA priming and foliar	1.00 ± 0.00	9.00 ± 0.00
	Drought x Zn priming	1.00 ± 0.00	2.00 ± 0.00
	Drought x Zn priming and foliar	2.00 ± 0.00	11.00 ± 0.00
***p* - values**			
W		$p < 0.05$	$p > 0.05$
T		$p > 0.05$	$p > 0.05$
W xT		$p > 0.05$	$p > 0.05$

The number of new bands statistically differed ($p < 0.05$) between the two water regimes (Table 19).

The number of lost RAPD bands detected in Zn priming was statistically significant different ($p < 0.05$) from the remaining treatments based on the Equality of Variances F test (Table 19). However, regarding the ANOVA report, no statistical significant differences ($p >$

0.05) were considered among treatments for both new and lost bands (Table 19). No statistically significant differences ($p > 0.05$) for the interaction water regimes x treatments were found (Table 19).

4. DISCUSSION

The Portuguese bread wheat cv. 'Jordão' was used in this work due to the following reasons: (i) bread wheat constitutes one model plant species with high economical and agricultural importance worldwide; (ii) this cultivar presents good agronomic performance and genetic homogeneity (required for CNV inscription) being useful for the detection of polymorphic patterns induced by the tested water regimes or treatments since a high degree of monomorphism among plants was expected.

Some genotoxicity studies did not describe extensively the patterns observed in the control plants (Liu *et al*. 2007; Cenkci *et al*. 2009; Kekec *et al*. 2010). However, in this work, it was detected polymorphism among the control plants of the bread wheat cultivar 'Jordão' in both watering and drought regimes. Although this bread wheat cultivar should be genetically homogeneous to be inscribed on the CNV, it preserved some degree of genetic variability which may be responsible for its high agronomic performance, adaptation potential and resistance to diseases. Besides, the polymorphism detected among the control plants of the drought regime may be a consequence of this abiotic stress. Overall, the DNA markers used in this study demonstrated to be highly sensitive and effective in the determination of genetic variation among individuals of the cultivar. As explained previously, PCR based markers are able to detect polymorphisms among individuals of the same population or species due to the direct examination of variation in the DNA sequences (Poczai *et al*. 2012). The level of genetic variation within populations is determinant for their evolutionary rate (Poczai *et al*. 2012).

Plant genotypes with better agronomic performance and/or ecological adaptation are expected to survive and reproduce better by shifting the gene pool over time, increasing the frequency of alleles responsible for the most successful genotypes (Ward *et al*. 2008). Genetic variability and diversity determine the responses of a given organism to abiotic and biotic stresses and natural selection, constituting important factors for evolution and definition of strategies for resources conservation and agriculture (Poczai *et al*. 2012). Hence, several studies based on different molecular marker systems have been performed in order to the genetic variability in plants under abiotic stress (Atienzar *et al*. 1999; Liu *et al*. 2005, 2007; Patade *et al*. 2005; Atienzar and Jha 2006; Guo *et al*. 2006; Enan 2007; Cenkci *et al*. 2009; Kekec *et al*. 2010; Voronova *et al*. 2011, 2014; Deshmukh *et al*. 2012; Sen *et al*. 2012; Fan *et al*. 2014; Hamat-Mecbur *et al*. 2014; Sigmaz *et al*. 2015; Sen *et al*. 2017). According to

Cenkci *et al*. (2009) and Kekec *et al*. (2010), the polymorphism revealed by the different DNA markers reflect the genomic instability of plants under stress relative to the control plants. Independently of the DNA marker used or type of stress under consideration, the polymorphic patterns comprised the newly appeared and lost bands relative to control plants, and reflected genomic instability caused by the stress (Liu *et al*. 2007; Cenkci *et al*. 2009; Kekec *et al*. 2010; Alzohairy *et al*. 2014; Cabo *et al*. 2014a,b).

Since in this work polymorphism was found among the control plants of each water regime, only the monomorphic bands (named as 'normal bands') were considered for comparison of patterns with the treated plants. The percentage of SCoT, CDDP, IRAP, REMAP, iPBs, ISSR and RAPD polymorphism achieved in all treatments and in both water regimes was considered genomic instability and the determination of the lowest values, enabled to extrapolate about the most adequate treatment(s) to be used in bread wheat under drought.

4.1. Genomic instability assessed by gene-targeted markers

In both watering and drought regimes, the number of polymorphic CDDP bands was higher than that of SCoT markers in both water regimes but more pronounced in drought. Plants under drought treated with AsA priming showed the lowest percentages of SCoT and CDDP polymorphism, hence, less genomic instability. In the case of Zn-treated plants, the CDDP markers detected lower percentages of polymorphism in plants under watering and under drought both treated with Zn priming, whereas SCoTs detected less genomic instability in plants of both water regimes treated with Zn priming plus foliar spraying. These results corroborated previous studies reporting the usefulness of Zn priming (Harris *et al*. 2008) and Zn foliar spraying (Karim *et al*. 2012) in the mitigation of drought effects in wheat, whereas other authors reported the usefulness of AsA priming (Malik and Ashraf 2012; Farooq *et al*. 2013; Wang *et al.* 2014), AsA on rooting medium and by foliar spraying (Malik and Ashraf 2012). However, it should be noticed that these previous studies did not use the same techniques as those of the present work.

SCoT markers detected lower genomic instability in plants of both water regimes treated with Zn priming plus foliar spraying. The different results achieved with SCoTs and CDDPs may be explained based on the choice of their primers once for the CDDP markers

amplification, primers specific for water stress responsive genes were selected. The SCoT primers selected for this work probably did not target gene regions involved in drought responses such as those targeted by the selected CDDP primers. Therefore, we considered the CDDP data more reliable in the extrapolation of the most suited treatment for bread wheat under drought.

Any type of conserved gene region or plant gene family can be tagged by CDDP markers (Poczai *et al*. 2012). Collard and Mackill (2009b) described a set of primers that target well characterized plant genes involved in responses to abiotic and biotic stress or plant development. For this study it was selected primers for water stress responsive genes previously isolated in bread wheat and whose sequences were previously published by other authors in the GenBank database. The WRKY genes *TaWRKY1*, *TaWRKY33* and *TaWRKY44*, are transcription factors involved in drought tolerance, by promoting the transcription of several defense-associated genes (Wang *et al*. 2015). *TaWRKY1* and *TaWRKY44* genes act as a positive regulator in drought stress response and are transcribed under abiotic stress situations. The *TaWRKY33* gene promotes drought tolerance by increasing the synthesis of abscisic acid (ABA) (He *et al*. 2016). MYB proteins act as transcription factors and mediate signal responses, playing significant roles in plant development, metabolism and stress response (Liu *et al.* 2011).

SCoTs have been widely used in the last years for genetic diversity assessment and DNA fingerprinting analyses in several plant species and hybrids (Cabo *et al*. 2014a; Atia *et a*l. 2017; Bhawna *et al*. 2017). The present work demonstrates their usefulness for detection of genomic instability in plants under stress. However, SCoT presented lower percentages of polymorphism than CDDPs. The highest total percentage of SCoT polymorphism was 73% and it was determined in bread wheat plants under drought and primed with Zn. This value indicated high genomic instability of the plants that could be attributed to drought. Nevertheless, a lower percentage of SCoT polymorphism (33%) was also observed in plants under drought but treated with Zn priming plus foliar application, indicating its suitability as drought antagonistic treatment in bread wheat. In fact, watered plants treated with Zn priming plus foliar application showed the lowest percentage of polymorphism achieved in this work (4%).

Both SCoT and CDDP techniques have been classified as targeted fingerprinting or gene-targeted markers due to their high reproducibility and targeting of regions throughout the plant genome being able for the development of phenotypically linked functional markers

(Poczai *et al*. 2012, 2013). Hence, the further isolation, sequencing and characterization of the new SCoT and CDDP bands detected in plants under drought might allow the identification of functional markers linked to phenotypes responsive to water stress in bread wheat.

4.2. Genomic instability revealed by RTNs-based markers and ISSRs

The genomic constitution and organization should be also considered in the interpretation of the RTNs-based and ISSR markers achieved.

Plant genomes are mostly composed of RTNs (Bennetzen *et al*. 1998; Shirasu *et al*. 2000; Wicker *et al*. 2001; Makarevitch *et al*. 2015). So, we should also consider that the whole plant genome responds to stress by involving the activation of transposable elements (Matzke and Matzke 1998; Alzohairy *et al*. 2014), elimination of highly repetitive or low-copy number DNA sequences (Feldman *et al*. 1997; Liu *et al*. 1998; Salina *et al*. 2000).

Previous studies reported that RTNs were activated due to abiotic stress, namely, *Tam* elements in snapdragon (Coen *et al*. 1986), *BARE-1* in barley (Kalendar *et al*. 2000) and Miniature Inverted-repeats Transposable Elements (MITE) in rice (Jiang *et al*. 2003). Therefore, the insertional activity of RTNs generates polymorphism and genomic changes that can be detected by the use of RTNs-based markers (Fan *et al*. 2014). This category of molecular markers has been useful for the analysis of genome structure (Schulman *et al*. 2012); biodiversity and functional genomics (Kang and Kang 2008; Soorni *et al*. 2013); genotoxicity (Hamat-Mecbur *et al*. 2014); genetic diversity (Branco *et al*. 2007; Carvalho *et al*. 2010, 2012; Nasri *et al*. 2013; Pandotra *et al*. 2013); and DNA fingerprinting (Muhammad and Othman 2005; Smýkal 2006; Cabo *et al*. 2014b; Delgado *et al*. 2017).

Since RTNs activation is rapidly induced under stress conditions (Grandbastien 1998; Alzohairy *et al*. 2014; Hamat-Mecbur *et al*. 2014), the use of RTNs-based markers in plants under stress can evidence the gain and/or loss of bands as demonstrated in this work. In all treatments, mostly in treated plants under drought, IRAP, REMAP and iPBS marker systems detected a high number of lost bands that probably arose from the insertional activity of RTNs. Indeed, Muhammad and Othman (2005) established a relation among polymorphic patterns, stress and RTNs activation after verifying that IRAP polymorphism was detected in individuals with an increased number of copies and activity of RTNs. RTNs are rapidly activated by stress and the visualized polymorphism in treated plants relative to control can be

a result of RTNs insertion throughout the genome inducing length polymorphisms. The variation in the length of the DNA sequences may change the location of primer binding sites bringing them closer and generating new bands, or it may widen the distance between them, preventing amplification and resulting in lost bands. Based on these assumptions, the involvement of RTNs should also be taken into consideration in the analyses of SCoT and CDDP data because the selected primers targeted plant genes with probable active RTNs flanking their binding sites, and RTNs activation under drought may also be responsible for the appearance and disappearance of CDDP and SCoT bands.

The IRAP markers revealed a higher number of lost bands in all treatments and both water regimes suggesting high genomic instability detected at the level of the LTR regions and between them. REMAPs also showed polymorphism values similar to those of IRAP, indicating that under drought, LTR and also SSR regions could be prompt to rearrangements that may result in the observed polymorphic patterns. As reported earlier, *A. thaliana* under drought showed SSRs stability (Yao and Kovalchuk 2011) favouring the annealing of SSR primers for ISSRs amplification. ISSRs demonstrated to be successful in the identification of markers related to drought tolerance in sugarcane (Patade *et al*. 2005), wheat (Deshmukh *et al*. 2012) and sugar beet (Sen *et al*. 2012), among others.

However, in this work, the ISSR markers were produced only with the primer SSR 8081, explaining the reduced or null polymorphism across the treatments of both water regimes. Similarly, despite we have tested various PBS primers, only the F0100 primer showed successful amplification, also explained the reduced or null iPBS polymorphism throughout the treatments of both water regimes.

One possible explanation for the absence of ISSR or iPBS polymorphism in some of the treatments could be the success of the drought antagonist tested that may reduce the genomic instability in these target regions and/or in the priming binding sites that generate them. This explanation would also reflect a fast response of SSRs and LTR regions to the treatment. The exception was 100% of iPBS polymorphism in the Zn-treated watered plants. This result may be due to a reduced number of monomorphic bands among the control plants produced by the single primer F0100, and to the few polymorphic bands in the treated plants, resulting on 100% of polymorphism.

High genomic instability was expected in control plants under drought (untreated). Nevertheless, it was higher in the watering regime, namely, in plants treated with AsA- and Zn-priming plus foliar application.

The plants under drought that showed lower values of polymorphism and hence, reduced genomic instability, were those treated with Zn- and AsA-priming. Based on the information displayed by the RTNs-based markers, Zn seems to be a better antagonist of drought effects in bread wheat than AsA.

For AsA- and Zn-treatments, the IRAP, REMAP and/or iPBS markers presented highest percentages of polymorphism in watering rather than in drought. This high genomic instability verified in watering could only be explained by plant responses to the treatments performed. This result may be due to the use of AsA and Zn dosages higher than those required by the plants. Other authors used AsA concentrations ranging from 0.5 mM to 1 mM in wheat (Al-Hakimi and Hamada 2001; Azzedine *et al*. 2011; Malik and Ashraf 2012). Therefore, in future tests, AsA could be applied in concentrations lower than 2 mM in seed priming and lower than 1 mM in foliar spraying as done in this work.

Zn is a micronutrient required by plants in low amounts but when provided in excess it can be toxic (Li *et al*. 2013). Other authors successfully used seed priming with 0.3% and 0.4% of Zn in bread wheat (Harris *et al*. 2008), which were similar to that used in our seed priming experiments. Besides, the concentration of 1.7 mM $ZnSO_4.7H_2O$ applied as foliar spraying corresponded to 0.1% of Zn which was lower than Zn concentrations previously used in bread wheat (Harris *et al*. 2008). The suitability of the Zn concentrations used in the present work may explain why most of the molecular data achieved here with different markers systems seem to suggest Zn as the most suitable drought antagonistic treatment to bread wheat.

4.3. Genomic instability determined by RAPD markers

RAPD assays are an important strategy to screen the genomic DNA. Indeed, these markers were already successful in the detection of several types of DNA damage and mutation in plants (Atienzar *et al*. 1999; Enan 2007; Liu *et al*. 2007; Cenkci *et al*. 2009; Kekec *et al*. 2010; Rashed *et al*. 2010; Abdalla 2011; Deshmukh *et al*. 2012).

In the present work, the changes in the genomic DNA induced by drought or by the applied treatments were detected in the RAPD profiles reflected by the disappearance or appearance of bands in treated plants relative to the untreated (control) ones. A similar approach was followed previously by other authors in bean plants exposed to Hg, B, Cr and

Zn which revealed polymorphic RAPD patterns relative to the control (Cenkci *et al*. 2009; Kekec *et al*. 2010). These authors considered the gain and loss of RAPD bands as genomic instability caused by the abiotic stress.

In the watered plants, the percentage of polymorphism achieved in both AsA treatments was the same (34.1%) whereas in the drought regime the percentage of polymorphism in AsA priming (43%) was higher than that registered in AsA priming plus foliar spraying (27.1%).

In both water regimes, the percentage of RAPD polymorphism was lower in plants treated with Zn priming (5.7% in watering and 11% in drought).

The percentages of polymorphism detected in Zn-treated plants were lower than those found in plants treated with AsA. Since the percentage of RAPD polymorphism was considered an evidence of genomic instability, the present results suggested that Zn treatments caused lower genomic instability in bread wheat under drought. Furthermore, between the two tested Zn treatments, the most effective for the mitigation of drought effects at the genomic level was Zn priming

Despite Zn appears to be a better drought antagonist, it is important to notice that in drought regime, the treatment AsA priming plus foliar application appeared to be more effective than AsA priming, since the former presented a lower percentage of polymorphism.

In the Zn-treatments and for both water regimes, the Zn priming plus foliar spraying showed higher percentages of polymorphism than Zn priming alone. This could be a sign that the treatment itself is causing some genomic instability, and as suggested before, the lowering of the Zn concentrations in future tests or applications should be taken into consideration.

Atienzar and Jha (2006) reported the effectiveness of RAPDs in genotoxicity studies and how these markers may complement cytotoxic analyses. These authors reinforced that RAPDs are sensitive to detect variations in bands intensity, gain/loss of bands as consequence of DNA damage induced by reduced concentrations of toxicants comparatively to those required for cytological analyses. Moreover, our research group has been developed cytogenetic analysis in bread wheat ‘Jordão’ by using root-tips of seeds primed with variable concentrations of Zn (lower and higher than those used in this work). Concentrations of Zn higher than 0.2% induced chromosomal anomalies but seeds primed with 0.4% of Zn benefited some yield-related characters (Reis *et al*., unpublished data). Hence, the exposure of seeds to a concentration of 0.4% Zn may induce genomic rearrangements detectable by

RAPDs, explaining the polymorphism percentages in watered Zn-treated plants. Nonetheless, these markers indicated Zn-priming as a suitable antagonist of drought effects in bread wheat.

4.4. Genomic instability: causes and consequences

In molecular studies performed in different plant species under abiotic stress with distinct marker systems, the detection of polymorphism such as the appearance of new bands and loss of bands relative to the control plants has been considered an evidence of genomic instability.

Recombination frequencies and mutation rates are high in plants under stress indicating that this condition rise genetic polymorphism (Nevo 2001).

Independently of the marker system used, the genomic instability revealed by the DNA markers may arise from different causes: DNA damage (e.g. single and double strand breaks), abasic sites, modified bases, oxidized bases, 8-hydroxyguanine, bulky adducts, point mutations and/or chromosomal rearrangements (Liu *et al*. 2007; Cenkci *et al*. 2009).

The case of disappeared bands may be explained by the occurrence of DNA damage or mutations that cause lesions in the DNA strand altering the PCR kinetics, and probably lead to the dissociation of the enzyme/adduct complex (Liu *et al*. 2007; Cenkci *et al*. 2009).

The newly appeared bands may arise from new annealing events that occur due to mutations within the priming binding sites, to large deletions that bring pre-existing annealing sites closer, and to homologous recombination (Liu *et al*. 2007; Cenkci *et al*. 2009).

Since a polymorphic band corresponding to mutant DNA sequences began to become apparent at a concentration of 1/50 of non-mutant DNA, a new band can appear if at least 2% of the DNA is affected (Jones and Kortenkamp 2000; Atienzar and Jha 2006) Therefore, in terms of probability of occurrence, rearrangements are the main factors influencing molecular profiles, particularly, those that lead to newly appeared bands. Point mutations can also influence molecular patterns but are less probable to occur in priming binding sites relative to rearrangements (Atienzar and Jha 2006).

On the other hand, events such as gene amplification and DNA methylation are also capable of altering the molecular markers profiles. Hence, the combination of all events at the

DNA level leads to greater changes than those due to individual lesions (Atienzar and Jha 2006).

In addition to the polymorphisms derived from alterations in the DNA sequence, factors inherent to the PCR mixture should also be considered like the selected *Taq* DNA polymerase (Song *et al*. 1995; Wendel *et al*. 1995; Matzke and Matzke 1998), the concentration of $MgCl_2$ or the amplification conditions that also dictate the specificity of the PCR. In this work, the CDDP, SCoT, IRAP, REMAP, iPBS, ISSR and RAPD markers were all amplified with the same *Taq* DNA polymerase (Thermo Scientific) which do not has a 3'→5' proofreading ability that may affect the DNA polymerization during the PCR. However, all reactions included a minimal concentration of 1.5 mM of $MgCl_2$, long primers, and high annealing temperature, except for RAPDs, ensuring a high specificity.

4.5. Integrated statistical analyses of all molecular data

According to Quinn and Keough (2002), the PCoA is similar to the Principal Components Analysis (PCA), but the former might extract results from a wide range of data such a genetic distance matrix.

Here, several PCoAs were performed based on the genetic distance matrices constructed with the pooled data produced by each marker system in both water regimes but only four are presented.

The following four PCoAs were constructed based on genetic distance matrices calculated with the pooled IRAP (Figs. 17 and 19) and RAPD (Figs. 18 and 20) data achieved in AsA (Figs. 17 and 18) and Zn (Figs. 19 and 20) treatments of both water regimes.

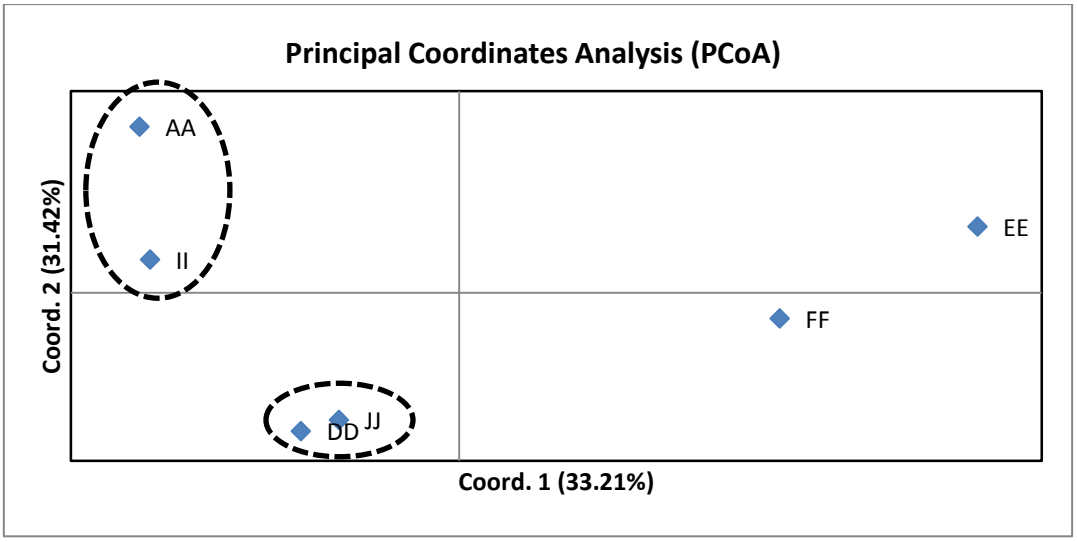


Figure 17. PCoA based on the genetic distance matrix calculated with the pooled IRAP data achieved in AsA-treated plants of both water regimes showing the close projection of watered control plants (AA) and plants under drought treated with AsA priming (II), as well as the close projection of watered plants treated with AsA priming (DD) and plants under drought treated with AsA priming + foliar spraying (JJ). Note: (EE) - watered plants treated with AsA priming + foliar spraying; (FF) – control plants under drought.

The PCoA of Fig. 17 projected the watered control plants (AA) in the same quadrant of plants under drought and treated with AsA priming (II), suggesting common molecular patterns among them, and hence, reduced genomic instability, as well as the suitability of AsA priming for drought effects mitigation (Fig. 17), corroborating the results achieved based on the percentage of polymorphism. Besides, it was also observed a close projection of watered plants treated with AsA priming (DD) and plants under drought treated with AsA priming + foliar spraying (JJ) (Fig. 17).

The first three axes of the PCoA based on the genetic distance matrix calculated with the pooled IRAP data achieved in the AsA-treated plants of both water regimes explained 88.26% of the molecular variation among the studied plants.

The IRAP results suggested that plants under drought when treated with AsA by seed priming and/or foliar spraying increase their genomic stability being similar to that of watered plants under control conditions.

The pooled RAPD data achieved in AsA-treated plants of both water regimes were used for the construction of the PCoA presented in Fig. 18.

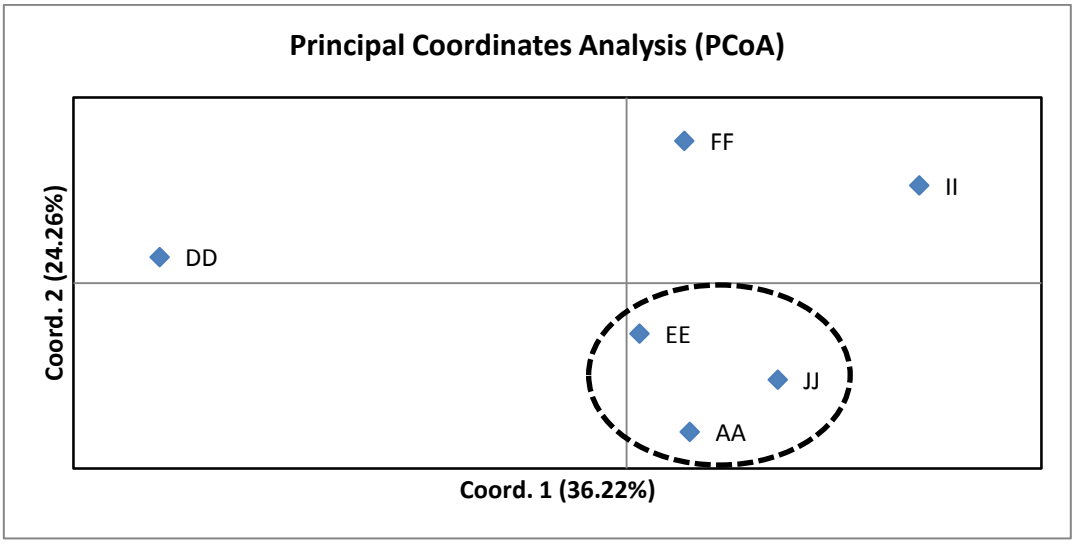


Figure 18. PCoA based on the genetic distance matrix calculated with the pooled RAPD data achieved in AsA-treated plants of both water regimes showing the close projection of watered control plants (AA), watered plants treated with AsA priming plus foliar spraying (EE) and plants under drought treated with AsA priming plus foliar spraying (JJ). Note: (DD) – watered plants treated with AsA priming; (FF) – control plants under drought; (II) – plants under drought treated with AsA priming.

In the same quadrant it were projected the watered control plants (AA), the watered plants treated with AsA priming plus foliar spraying (EE), and plants under drought treated with AsA priming plus foliar spraying (JJ) (Fig. 18), corroborating the previous results based on the percentage of polymorphism. This result may arose from the occurrence of common RAPD patterns among these plants, evidencing that AsA priming + foliar spraying increased the genomic stability in plants under drought (JJ) similar to the watering control (AA) and watered plants treated with the same method (EE).

The three first axes of this PCoA explained 76.11% of the total molecular variation found among the studied plants.

The PCoA based on the genetic distance matrix constructed with the pooled IRAP data achieved in Zn-treated plants of both water regimes showed a close projection of the watered control plants (AA) with plants under drought and treated with Zn priming (GG) (Fig. 19), corroborating the results obtained with the percentage of polymorphism.

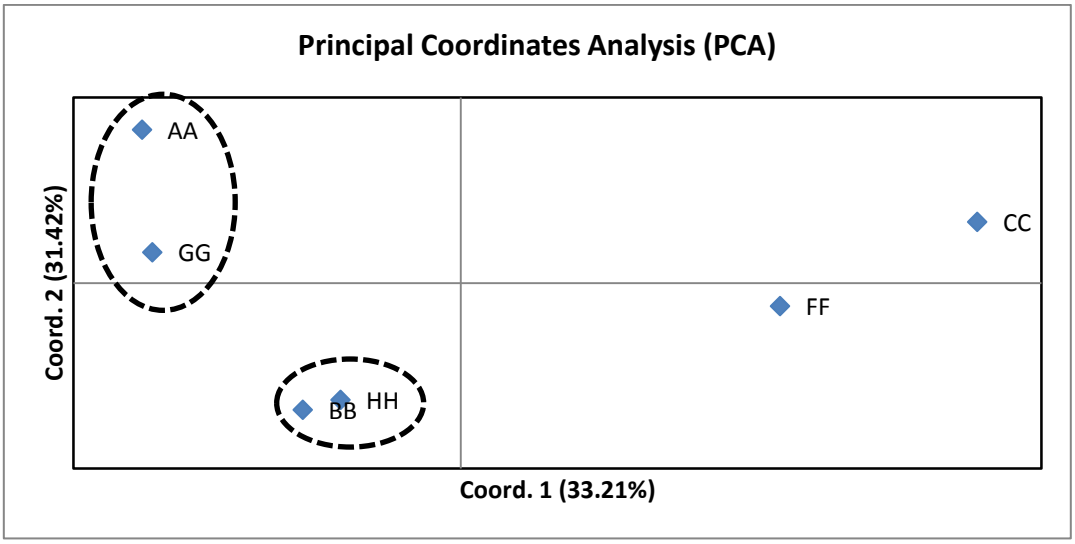


Figure 19. PCoA based on the genetic distance matrix calculated with the pooled IRAP data achieved in Zn-treated plants of both water regimes showing the close projection of watered control plants (AA) and plants under drought treated with Zn priming (GG), as well as among watered plants treated with Zn priming (BB) and plants under drought treated with Zn priming + foliar spraying (HH). Note: (CC) – watered plants treated with Zn priming + foliar spraying; (FF) - control plants under drought.

The first three axes of the PCoA presented in Fig. 19 explained 88.26% of the total molecular variation found among the studied plants.

In Fig.19 we can also observe a close projection of watered plants treated with Zn priming (BB) and plants under drought treated with Zn priming + foliar spraying (HH). However, this result was not in accordance with the achieved percentages of IRAP polymorphism determined previously, since watered plants treated with Zn priming showed higher genomic instability (57.78%) than plants under drought treated with Zn priming plus foliar spraying (22.86%). Although the exogenously application of Zn in plants under drought may decrease genomic instability, in watered plants the priming with high dosage of this micronutrient may induce stress. Again, the future test of Zn priming with lower dosage in seed priming is suggested.

The PCoA presented in Fig. 19 suggests that plants under drought when treated with Zn priming present were genetically closer to control plants of the watering regime. In fact the polymorphism percentage achieved in plants under drought treated with Zn priming was 15.94% being lower than that registered in watered plants treated with the same method. Hence, the IRAP markers evidenced the effectiveness of Zn priming and/or foliar spraying in bread wheat plants under drought.

The PCoA of Fig. 20 projected closely the watered control plants (AA), the watered plants treated with Zn priming plus foliar spraying (CC) and plants under drought treated with Zn priming plus foliar spraying (HH).

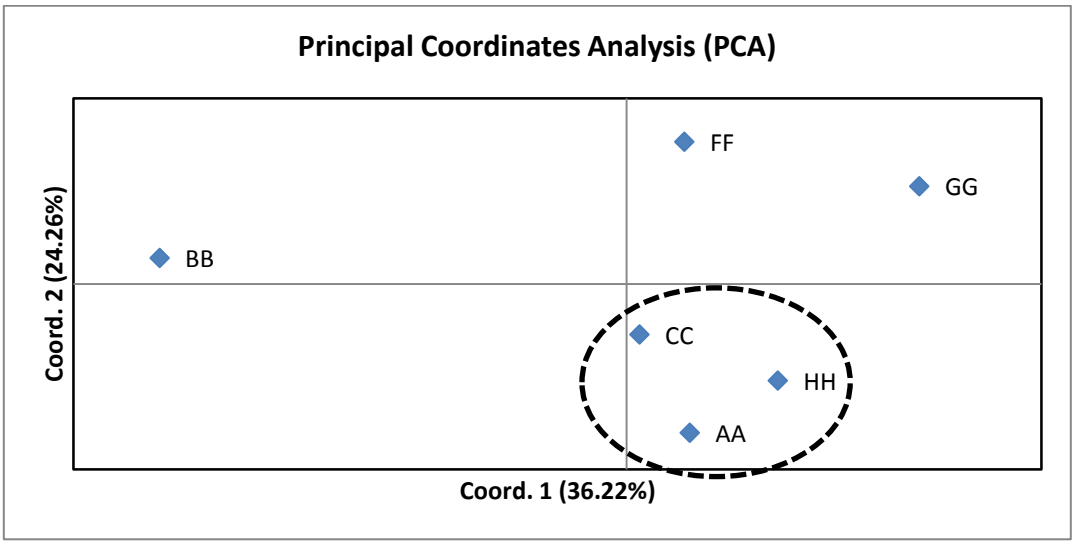


Figure 20. PCoA based on the genetic distance matrix calculated with the pooled RAPD data achieved in Zn-treated plants of both water regimes showing a close projection among watered control plants (AA), watered plants treated with Zn priming plus foliar spraying (CC) and plants under drought treated with Zn priming plus foliar spraying (HH). Note: (BB) – watered plants treated with Zn priming; (FF) - control plants under drought; (GG) - plants under drought treated with Zn priming.

The three first axes of the PCoA presented in Fig. 20 explained 76.11% of the total molecular variation among the studied plants.

The RAPD data achieved in Zn-treated plants of both water regimes suggest common molecular patterns and genomic stability among watered control plants (AA), watered plants treated with Zn priming plus foliar spraying (CC), and plants under drought treated with Zn priming plus foliar spraying (HH). These results were common to those achieved with IRAPs and indicated the effectiveness of Zn priming and/or foliar spraying in bread wheat under drought.

The common results achieved in AsA- and Zn-treated plants with IRAPs and RAPDs, two marker systems of different categories, might suggest the existence of primer binding sites for RAPDs within RTNs since the former constitute amplicons between LTRs of RTNs and their insertional and transcriptional activity may be also involved in the length polymorphisms detected by RAPDs and generation of gain and loss of bands.

Based on the genetic distance approach graphically represented by the PCoAs, both IRAPs and RAPDs demonstrated to be reliable in the genomic stability assessment among the studied plants under watering and drought and treated by both Zn and AsA treatments. However, IRAP can be more informative than RAPDs because the former markers explained higher percentages of total molecular variation by the cumulative sum of the percentages explained by the first three axes of each PCoA relative to the RAPDs-based PCoAs.

Although the existence of different interpretations for a PCoA, regarding the criterion reported by Reis (1997), it could be retained that the first five coordinates explain 70% of the total molecular variation, corresponding to a percentage value with statitiscal significance. Assuming such limit of cumulative percentage, both IRAP and RAPD techniques explained more than 70% of total molecular variation with the first three coordinates (Figs. 17 to 20).

The IRAP and RAPD markers were successfully used in previous studies of genomic instability assessment in plants under stress (Cencki *et al*. 2009; Kekec *et al*. 2010; Sigmaz *et al*. 2015). In this work, IRAPs and RAPDs produced the higher number of amplified bands as result of the use of a larger number of primers and/or primer combinations. Although it were performed, the remaining PCoAs based on the additional marker systems used in this work, did not reveal any type of specific projection of plants in terms of drought antagonistic treatment or water regime, and it were not presented.

In addition to IRAP and RAPD, the other marker systems used here were also efficient in detecting genomic instability. However, they sould be performed with a higher number of primers in order to produce a larger number of markers.

5. CONCLUSIONS

Both SCoT and CDDP gene-targeted markers allowed a genome-wide DNA variation analysis responding differently to the tested treatments.Both markers constituted a useful tool for detecting genomic instability in wheat plants under drought and could be extensible to other plant species.

Since CDDP markers were based on primers designed for water stress responsive genes, their lowest polymorphism levels per water regime and treatment may indicate that AsA- or Zn-priming alone could help to solve negative consequences of drought in bread wheat cultivation.

The IRAP and REMAP techniques evidenced higher percentages of polymorphism, corroborating the assumption that plants under abiotic stress such as drought have higher RTNs insertional and transcriptional activities that could be detectable by RTNs-based markers. The comparison among the different marker systems tested here, their target regions and primer annealing sites, allow us to argue that LTR regions, as well as genomic DNA between them and between LTR and SSR regions, are prompt to genomic rearrangements under drought. On the other hand, the regions between two PBS appear to be stable as evidenced by the low percentage of polymorphism the iPBS markers. However, this assumption needs to be clarified further by the use of a higher number of PBS primers.

With the information gathered by the RTNs-based markers, IRAP and REMAP, the best antagonist treatment for drought mitigation is seed priming with AsA or Zn, based on the most reduced percentage values of polymorphism.

Concerning to the analysis performed with the PCoA based on the pooled IRAP data, the genomic stability of plants under drought treated with AsA priming or Zn priming was similar to that verified in the watered control plants suggesting once more the effectiveness of seed priming with AsA or Zn as drought antagonistic treatments in bread wheat.

RAPD assays demonstrated to be a useful tool in order to do a genome wide analysis for the detection of genomic instability in bread wheat plants under abiotic stress. Indeed, the RAPD data also suggested that the best antagonistic treatment for drought was Zn priming based on the lowest percentage values of polymorphism. However, the PCoA results obtained in AsA- and Zn-treated plants based on the pooled RAPD data suggested as best drought antagonistic treatments, AsA priming plus foliar spraying, as well as, Zn priming plus foliar spraying.

The foliar application of AsA has been successfully used in plants under stress, and in some cases, it was applied more than once and in lower concentrations comparatively to the present study (Karim *et al*. 2012; Malik and Ashraf 2012). These features may explain why, based on the percentage values of polymorphism it was considered Zn as a better drought antagonist than AsA.

Overall, integrating all the results achieved in this work, seed priming with Zn (Zn priming) presented the lowest percentages of polymorphism more frequently, suggesting it as the most suitable drought antagonistic treatment for bread wheat. In addition to the diminishing of the negative impacts of drought in plant growth and development, Zn treatments have been reported as favorable for grain yield improvement and also can improve the nutritional value of bread wheat grain (Harris *et al*. 2008), which will be useful for the genetic breeding of 'Jordão'. Besides, Zn priming constitutes a cost-effective method that could be used in agronomic biofortification that produce fast results in contrast to the long term classic breeding programs involving genotypes selection and crosses.

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