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EFFECT OF PLOIDY LEVEL ON PLANT ABIOTIC STRESS RESPONSE IN WESTERWOLTHS RYEGRASS

Doctoral Dissertation

Agricultural Sciences, Agronomy (A 001)

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Olakunle Kelvin AKINROLUYO

PLOIDIŠKUMO ĮTAKA GAUSIAŽIEDĖS VIENAMETĖS SVIDRĖS ATSAKUI Į ABIOTINĮ STRESĄ

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INTRODUCTION

The research problem

Environmental stresses affect the productivity of many agricultural crops globally. Many regions of the world are experiencing prolonged droughts that threaten agricultural production. The world population is constantly increasing, and estimated to reach nine billion people in the year 2050; however, the land available for agricultural use is currently limited, and it is necessary to explore lands that have not been used for agricultural purposes, such as semi-arid regions and highly salinised soils in order to improve food production globally (Hussain *et al.,* 2009; Godfray *et al.,* 2010).

Drought and salinity are the main abiotic stresses that affect plant productivity. The threat posed by global warming is gradually becoming a reality, as extended periods of high temperatures were recorded in the northern parts of Europe in 2018 (UNCC, 2019). The frequency of various extreme climatological events is increasing due to climate change; all global climate models predict rising temperatures and consequently an even higher risk of drought in many areas of the world in the future, either due to a reduction in precipitation, an increase in evaporation, or a combination of these factors (Dai, 2012; Sherwood & Fu, 2014).

More than 6% of the global land area has severe salinity problems, and this translates to about 800 million hectares (Yadav *et al.,* 2011). Extreme drought requires irrigation farming practices which can result in increased soil salinity over a period of time (Qadir *et al.,* 2014). A good approach to food sustainability is to increase crop productivity in these suboptimal soil conditions, especially in highly salinised soils. The impact of climate change on staple food production is the main concern (Trnka *et al.,* 2015), and abiotic stresses affect forage production as well, pressing farmers and breeders to search for means of adapting the grasslands to arising challenges (Ergon *et al.,* 2018).

Polyploid plants have demonstrated good resistance to both biotic and abiotic stress, including drought and salinity (Xue *et al.,* 2015; Godfree *et al.,* 2017). This enables them to better adapt to a wider ecological region (Blanc & Wolfe, 2004). The increase in tolerance to abiotic stress can be attributed to a higher chromosome number and gene expression, causing increase in the synthesis of a proteins or secondary metabolites. The gene expressions in polyploids differs from that in their diploid counterparts, and some of the expression changes might be a result of the increase in the copy number of the chromosomes: this could affect all the genes equally, resulting in their uniform increase in expression level (Livak & Schmittgen, 2001). Polyploid plants can

demonstrate higher adaptability, increased vigour and resistance to unfavourable environmental factors compared to their diploid relatives (Sattler *et al.*, 2016), yet some studies suggest that diploids are more resistant to abiotic stresses (Sugiyama, 2006; Balocchi & López, 2009; Helgadóttir *et al.*, 2018) or that the differences are small (Kemesyte *et al.*, 2017).

Most research projects use plants with different pedigrees when exploring differences in yield, stress resistance and changes in transcriptome between diploids and tetraploids. This makes interpretation of the results more difficult – the changes in phenotypic traits or gene expression might be due to different genetic background instead of ploidy level. Comparing induced autotetraploids to their respective diploid parental lines helped to avoid this problem and gave more reliable insights into the effect of ploidy level on the performance of the plant.

Research aim

The aim is to evaluate the yield-related traits and resistance to abiotic stress of diploid and tetraploid Westerwolths ryegrass cultivars and induced autotetraploid lines, and to investigate the expression level of candidate genes involved in the response to abiotic stress in diploid and tetraploid plants.

Research objectives

1. Determine the optimal concentration of mitosis inhibitors for inducing tetraploids from 10 diploid cultivars of Westerwolths ryegrass.
2. Evaluate the yield-related traits and resistance to diseases of diploid cultivars and respective tetraploid populations in field experiments.
3. Evaluate the tolerance of diploid and tetraploid plants to salinity at the germination and vegetative stages.
4. Evaluate the tolerance of diploid and tetraploid plants to drought in controlled conditions.
5. Evaluate candidate gene expression levels in diploid and tetraploid plants compared to resistance to drought stress.

Defended statements

1. The ploidy level affects tolerance to drought stress in Westerwolths ryegrass.
2. The ploidy level and antiradical activity contribute to tolerance to salinity stress.
3. The gene expression level of functional proteins gives the induced tetraploid lines an advantage over the diploid progenitors in response to drought.

Research hypothesis

Diploid cultivars of Westerwolths ryegrass differ from induced autotetraploids in yield, resistance to salinity and drought. The difference in resistance to abiotic stress is caused by different gene expression levels between diploid and induced tetraploid plants.

Novelty of the research project

The response to drought and salinity has been extensively studied in the *Lolium* genus. Many of these studies were focused on Perennial and Italian ryegrass. Data on the abiotic stress resistance of Westerwolths ryegrass and the genetic factors behind it, let alone comparison between diploids and tetraploids, is scarce. In the study, we investigated the effect of ploidy on abiotic stress response using diploid cultivars of Westerwolths ryegrass and their respective induced tetraploid lines. The comparison of induced autotetraploids to their respective diploid parental lines provided a unique opportunity to purposefully evaluate the influence of ploidy on plant phenotypic traits and response to abiotic stress while avoiding discrepancies relating to different genetic backgrounds

Approval of the research work

Two papers were published in journals in the Clarivate Analytics Web of Science database. In addition, this research work has contributed to one international book chapter. The results of this work have been presented at four conferences.

Volume and structure of the work

This dissertation is written in English. It consists of the Introduction, Literature Review, Material and Methods, Results and Discussion, Conclusions, List of References, List of Publications, and Acknowledgements chapters. The dissertation comprises 142 pages and is illustrated with 12 tables and 18 figures. A total of 288 reference sources are cited in the dissertation.

1. LITERATURE REVIEW

1.1. Grasslands, an important ecosystem

Grasslands are a diverse ecosystem and they make up about 80% of the global agricultural area, and approximately 26% of the total global land area (Steinfeld *et al.,* 2006; Wright *et al.,* 2006), (FAO, 2008). Grasslands are one of the largest ecosystems in the world and include annual, biannual and perennial species. The population dynamics of grassland plants are often defined by the demography of species inhabiting a particular location, which is influenced by demographic parameters such as survival, life span, life expectancy and traits including the ability of seeds to germinate (Silvertown & Doust, 1993) .

Grasslands are vital in the sustenance of food production. 70% of both the world's beef and cow's milk are dependent on forage production in the grasslands (Lauenroth & Adler, 2008). Fodder and forage grasses play a significant role in the livestock industries. The production and quality of forage grasses directly affects the value of livestock products (Roy, 2009). Grasslands are also potentially effective in reducing greenhouse gas emissions (Soussana *et al.,* 2007). According to Lai (2004) and Powers *et al.* (2011), grasslands have an annual carbon sequestration potential of close to 0.3 billion tons of organic soil carbon, and counteract up to 4% of greenhouse gas emissions. The net amount of carbon sequestered in grassland soils varies between years, locations, grassland age and type (Byrne *et al.,* 2007). Net Carbon storage also varies with changes in annual temperature, radiation and rainfall (Hunt *et al.,* 2004; Soussana *et al.,* 2007).

1.2. Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*)

The *Lolium* genus is comprised of 10 species (Terrell, 1968). *Lolium perenne* L. and *Lolium multiflorum* Lam. are the most common forage ryegrass species, of which more than 150 Westerwolths ryegrass cultivars have been recognised in Europe and more than 500 cultivars of *Lolium multiflorum* have been registered (Spangenberg *et al.,* 2005; Humphreys *et al.,* 2010). A hybrid between *L. perenne* and *L. multiflorum*, known as *Lolium x hybridum* Hausskn (*Lolium boucheanum* Kunth), is also cultivated, although it is less common.

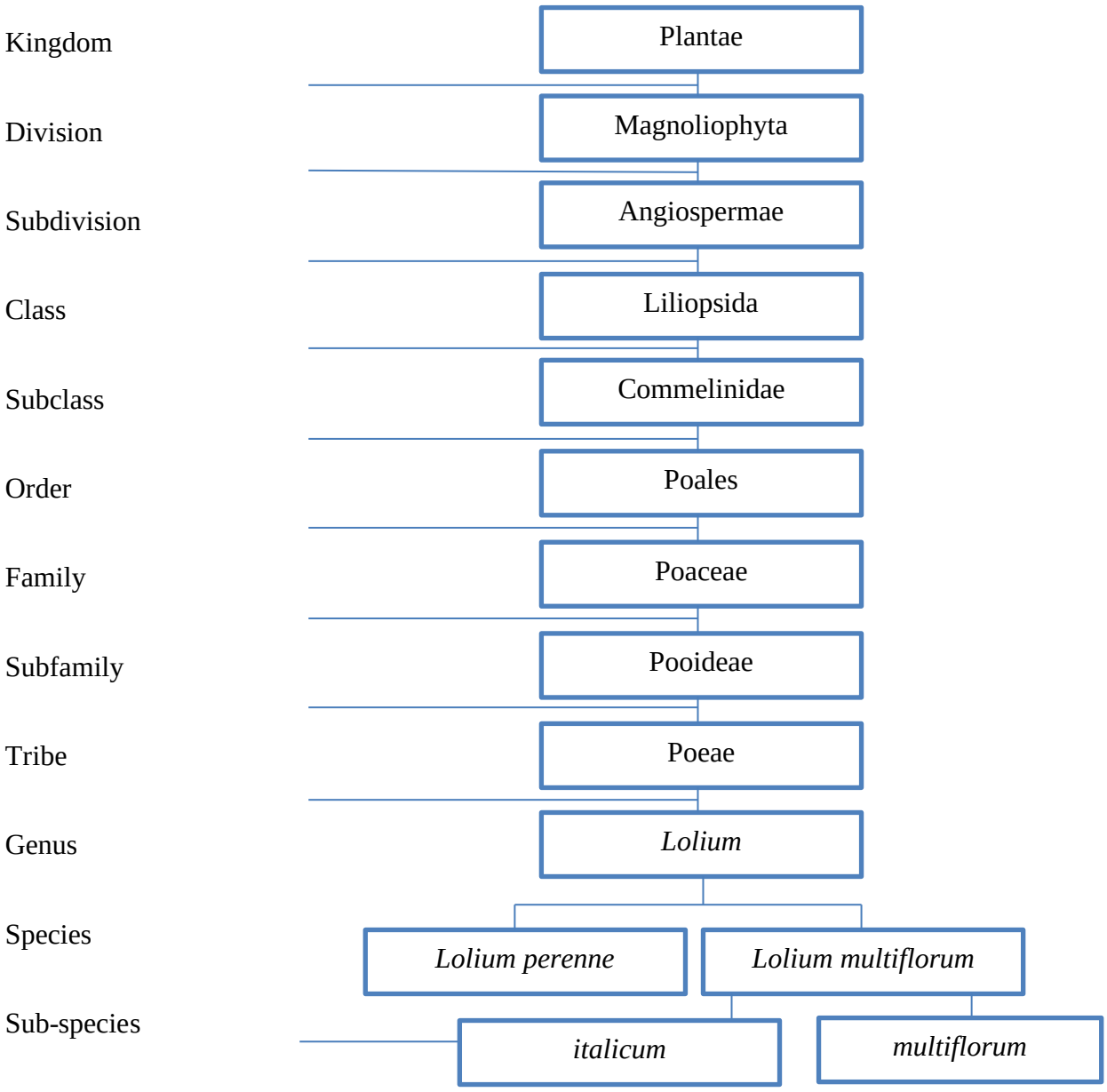


Figure 1. *The taxonomy of Lolium multiflorum spp multiflorum*

Lolium multiflorum spp *italicum* produces a high yield over a long growing season. It grows rapidly, and it is also suitable for grazing, and has been used for hay and silage production (Wilkins, 1991; Reheul *et al.*, 2003). Italian ryegrass is often included in permanent pasture mixtures to complement the slow-growing perennial ryegrass. It also provides good winter growth compared to perennial grasses with little growth in winter periods in Australia (Lamp *et al.*, 1990).

Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*) is a forage grass with high palatability and digestibility. It is fast growing due to its annual nature and produces high first cut yields. When spring-sown under continental conditions, Westerwolths ryegrass will produce high first cut silage yields in the summer (Humphreys *et al.*, 2010). It can be cultivated in pure stands or mixtures for

high-quality green feed or silage production. Westerwolths ryegrass is a reliable cool-season grass that has leaves that are rich in protein, vitamins and minerals, in addition to being highly digestible and palatable to grazing animals (Humphreys *et al.*, 2010). In addition to cultivation for silage production, Westerwolths ryegrass can also be used as a catch crop (Humphreys *et al.* 2010). Westerwolths ryegrass, as well as its close relative perennial ryegrass (*L. perenne*) and Italian ryegrass (*L. multiflorum* ssp. *italicum*), are self-incompatible and occur naturally as diploids (2 n = 2 x = 14). Westerwolths ryegrass grows taller than *L. perenne* and also has wider leaves, and longer glumes. Westerwolths ryegrass has longer inflorescence, more spikelets, more florets per spikelet and bigger seeds than *L. perenne* (Polok, 2007).

Like perennial ryegrass and Italian ryegrass, Westerwolths ryegrass is wind-pollinated and the propagation is mainly by seed. In some cases, annual ryegrass can self-pollinate and produce seed, however, the offspring are either not viable or are unfit as a result of the small number of caryopses (Beddows, 1973). Westerwolths ryegrass can serve as a good substitute forage plant for perennial ryegrass and Italian ryegrass, providing nutrients for ruminant animals. It is highly cultivated in grasslands due to its excellent forage qualities, thus forming the basis for a global grassland production system (Wilkins & Humphreys, 2003). It produces large amounts of seed when cultivated in optimal conditions, and one hectare can produce some 2000kg of clean seeds. Westerwolths ryegrasses are capable of growing in different soil types, except in very poorly or excessively drained soils, and at a lower pH limit of 4.5 (Humphreys *et al.*, 2010). It is versatile, easy to establish and adapted to a wide range of soil types. It produces high herbage yield (70% dry matter and 20% crude protein) and has high nutritional value (Gilliland *et al.*, 2002).

1.3. Climate change: a major contributor to abiotic stress

Although there is yet to be a general consensus on the impact of climatic change on the planet (Nisbet & Cooper, 2015), let alone its impact in contributing to increased abiotic stress, there is a variety of data confirming the devastating impact of climate change (Bastin *et al.*, 2019; Haines & Ebi, 2019). Humans have an enormous effect on the climatic system in terms of the production of greenhouse gasses (IPCC 2014). There has been a significant change in the average temperature and the amount of rainfall over the past 50 years (Jung *et al.*, 2002; Fauchereau *et al.*, 2003). The increase in temperature can be linked to the increase in the production of these greenhouse gases such as ozone (O_3), methane (CH_4), carbon dioxide (CO_2) water vapour, nitrous oxide (NO) and chlorofluorocarbons (Anderson *et al.*, 2016).

There has been an increase in the yearly mean concentration of CO_2 in the atmosphere from 320 $\mu mol \cdot mol^{-1}$ in 1965 to about 400 $\mu mol \cdot mol^{-1}$in 2017, and this increase continues rapidly

(Bunce, 2017). Some studies have shown that elevated CO_2 levels are beneficial for plant growth due to enhancing photosynthesis, especially in plants that utilise the C3 photosynthetic pathway (Drake *et al.,* 1997; Ziska *et al.,* 1997; Kim *et al.,* 2003). A combination of other factors such as changes in the ozone, water deficit, nutrient unavailability and increased temperatures, can counterbalance the potential for yield increase (Ainsworth *et al.,* 2012; Dias de Oliveira *et al.,* 2013; Ruiz-Vera *et al.,* 2013; Cai *et al.,* 2015; Kimball, 2016; Broughton *et al.,* 2017). Elevated CO_2 levels have also been linked to a reduction in the nitrogen and protein content in soya bean plants, leading to a decrease in yield (Li *et al.,* 2018).

Increases in temperature affect crop production both directly and indirectly. The impact of increased temperature depends on the crop's optimal temperature for growth and reproduction. An increased temperature could be beneficial for some plants, while posing threats to the cultivation of other crops and leading to a significant loss in yield (Schlenker & Roberts, 2009; Hatfield *et al.,* 2011; Urban *et al.,* 2012; Wheeler & von Braun, 2013). An increase in temperature can also hasten and prolong drought periods, and ultimately result in significant losses in crop yields. Many annual seed producing crops, including Westerwolths ryegrass, are cultivated in regions where temperatures are optimal or close to the optimal temperature for seed yield during the reproductive development, and therefore a significant increase in the temperature will negatively impact the seed yield even if there is an increase in the length of the growing season.

1.4. Drought, a limiting factor to crop yield

According to data from the United Nations water development report, global water consumption has tripled over the last 50 years. It has also been estimated that water demand for agricultural purposes accounts for more than two-thirds of global water use and continues to rise. This makes drought a serious concern (Johnson *et al.,* 2001).

Plants are faced with numerous sources of biotic and abiotic stress, which limit productivity. Drought is a major abiotic stress that reduces the vitality of plants and hampers plant productivity in many parts of the world (Flexas *et al.,* 2004; Fischer & Polle, 2010). It has a devastating effect on plants at all stages and is the biggest challenge for breeders. Drought is the main threat to world food security and has been the main cause of food shortages in the past (Farooq *et al.,* 2009). Heisey and Edmeades (1999) reported a yield loss of approximately 24 million tons of maize annually due to drought stress.

Water is essential for plant growth and development, and makes up some 80-95% of the total mass of growing plant tissue (Chen *et al.,* 2015). Absorbed water is moved to other plant tissues, such as leaves, via the xylems, to facilitate processes such as photosynthesis. One of the first

responses to drought in plants is stomatal closure, and this causes a reduction in the photosynthetic rate and also a reduction in the evapotranspiration process (David *et al.,* 2007; Chaves *et al.,* 2009; Lawlor & Tezara, 2009). This results in a decline in photoassimilate production, and the distribution of the photoassimilate is also altered, with root growth favoured over shoot growth in order to maximise the uptake of water from the soil (Sharp, 2002). Biomass production (leaf and shoot elongation) and seed yield are drastically affected during water deficit conditions.

Drought intensity depends on many factors such as the frequency and distribution of rainfall, soil types (water holding capacity), and temperature, and in essence, its severity can be unpredictable. Rain-fed ecosystems are the most prone to mild, moderate and severe drought. Yields are affected through various routes at different growth stages, and at different intensities, in drought (Pantuwan *et al.,* 2002; Pirdashti *et al.,* 2009). Losses resulting from drought are not only limited to agricultural production losses, but include ecological damage, soil erosion and land desertification, which means that drought should be considered an urgent global and environmental constraint. This has recently prompted many countries and international organisations to launch research projects that explore water-saving mechanisms and drought resistance in plants, in order to identify superior cultivars.

Drought affects the physiology and morphology of plants. Mild drought tends to increase root depth in the soil (Fayez & Bazaid, 2014; Kunert *et al.,* 2016). In Westerwolths ryegrass, as in any other crop, the sugars are mainly produced in the green tissues. The leaves, which are the main site for photosynthetic activities, play a vital role in seed production. The flag leaves are known to produce a significant proportion of the carbohydrate reserves in seed (Praba *et al.,* 2009). Reports have shown that the flag leaves are shorter in less irrigated plants and this contributes mainly to a reduction in grain yield (Todaka *et al.,* 2017).

1.5. Drought tolerance in plants

When plants grow satisfactorily in water deficit conditions, they are defined drought-tolerant (Mitra, 2001). Plant breeders further defined drought resistance in terms of the relative yield of genotypes, that is, the ability of a plant to produce an economic yield with minimal loss in water deficit conditions (Tardieu *et al.,* 2018). Plants growing in different habitats have evolved several different resistant strategies that enable them to grow and thrive in conditions that are not suitable for other plants. They develop traits that enable them to adapt to unfavourable conditions. These traits range from changes in anatomical structure and plant morphology to biochemical and physiological responses. The change from vegetative growth to reproductive growth can be accelerated or delayed when drought becomes severe (Pettigrew, 2004).

Drought tolerance is an important trait and is related to yield trait. Plants respond to drought stress via four mechanisms (Figure 2): shoot dehydration avoidance, drought tolerance, drought escape and drought recovery (Yue *et al.*, 2006; Luo, 2010; Lawlor, 2013). Of these four mechanisms, shoot dehydration avoidance and drought tolerance are the two major mechanisms conferring resistance to drought in plants.

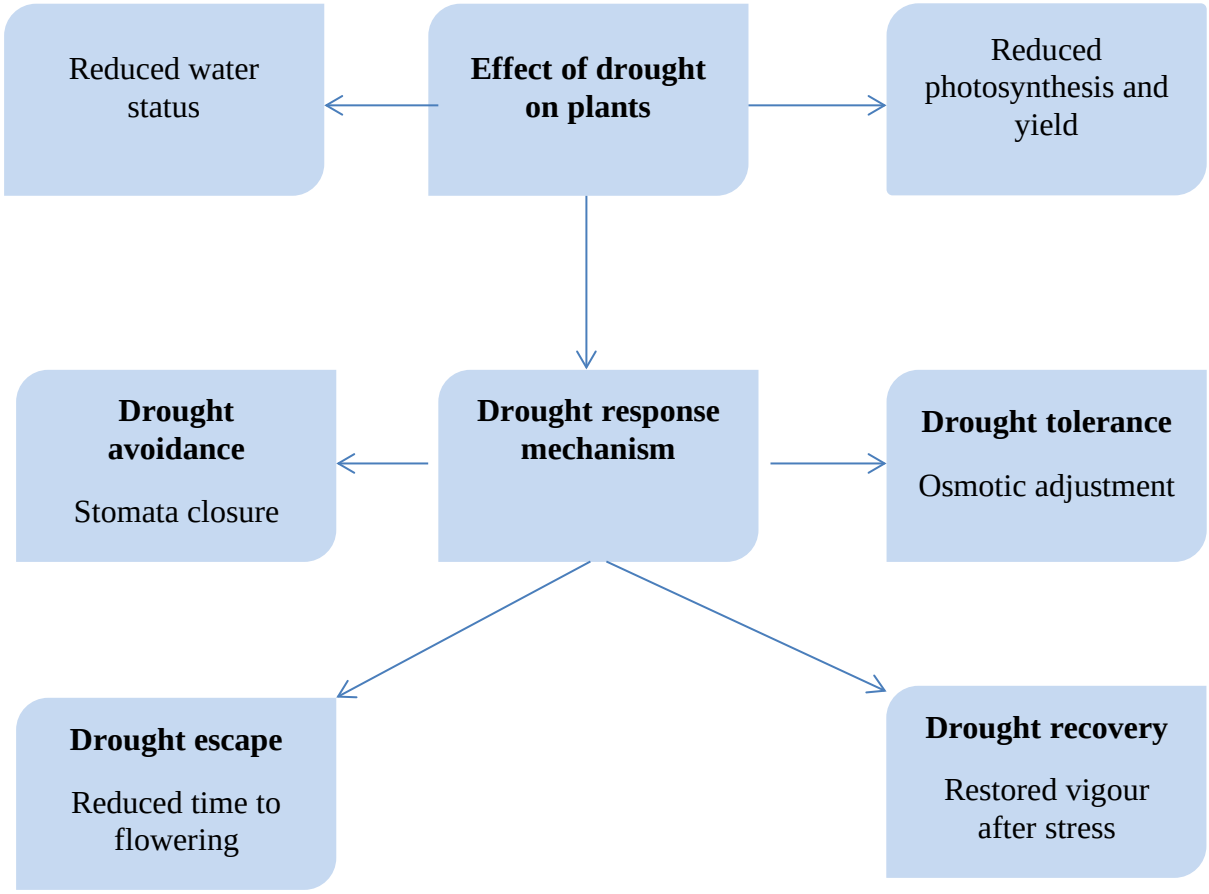


Figure 2. *General scheme of plant response to drought (modified from Fang & Xiong, 2015)*

1.5.1. Shoot dehydration avoidance-associated mechanisms

Shoot dehydration avoidance, also referred to as drought avoidance, occurs when plants are capable of maintaining their normal physiological processes during mild drought stress by modifying some of their morphological structures to counteract the negative effect resulting from the stress (Blum, 2005). Broadly, shoot dehydration avoidance occurs when plants slow down their growth rates. It involves various morphological and anatomical changes that reduce the loss of water through transpiration and also improve the uptake of water from the soil via modulation of root system architecture (Luo, 2010). One of the main attributes of plants during shoot dehydration avoidance is their ability to maintain high water potential. Plants achieve this through

the rapid closure of the stomata, leaf rolling and an increase in leaf waxing on the surface of the leaves to prevent water loss (Tardieu *et al.,* 2014). The guard cells that surround the stomatal pores are very sensitive to changes in environmental conditions. When guard cells receive environmental stimuli, the opening and closing of the stomata are controlled by the changes in the turgor and water potential in the guard cells; this regulates pivotal physiological processes in the plant (Li *et al.,* 2006). Drought tolerant plants close the stomata before the leaf water status involves wilting (Fang & Xiong, 2015). Stomata respond to drought through two main mechanisms. Firstly, they respond to the air humidity, which triggers the guard and epidermal cells to evaporate moisture, prompting the closure of the stomata (Trenberth *et al.,* 2013). Secondly, the stomata respond to the changes in water potential in the leaves, and shut when the leaf water potential drops below the threshold (Tripathi *et al.,* 2015).

Physiological and morphological changes in the leaves play an important role in promoting water use efficiency, and reducing water loss. Wilting, a phenomenon in which leaves roll due to the loss in the cells' turgor pressure occurs when plants sense severe water deficiency (Fang & Xiong, 2015). Wilting is a drought adaptive trait which is triggered by turgor pressure. It prevents excessive water loss during drought conditions. In some plants, wilting occurs during the day when the rate of transpiration is high, and they gradually unfold or unroll at night when there is a reduction in water loss (Saglam *et al.,* 2014). Some plants change the orientation of leaf blades to become parallel to the direction of light, usually by rolling (Baret *et al.,* 2018). This active and passive movement in the leaves plays a role in minimising the incident solar radiation, helping to reduce the surface temperature in leaves and preventing excess water loss from the plant (Ambavaram *et al.,* 2014). The leaves of plants growing in semi-arid regions have xeromorphic structures such as thicker and smaller leaves, and the stomata are denser and smaller. These plants also have thicker cuticle epidermis and more developed vascular bundles of sheath (Abdulraham & Oladele, 2011).

Plants use their roots to absorb nutrients and water from the soil, and therefore roots play a vital role in responding to mild and severe water deficiencies. Plants can enhance their ability to absorb water from the soil through a well-developed root system by increasing their root to shoot ratio, their rooting density or their rooting depth. Some plants sense drought early and have the ability to increase root growth during this early stage of stress to enable them to absorb water more deeply from the soil (Hu & Xiong, 2014). Some studies have shown that the weight, volume, length, and density of plant roots are associated with drought resistance in crops (Kamoshita *et al.,* 2002; Price *et al.,* 2002; Hammer *et al.,* 2010). A positive correlation has been found between how deeply roots penetrate the soil and drought resistance in *Phaseolus acutifolius*

(Mohamed *et al.,* 2002). In arid regions, seedlings from woody plants have vertical roots that are 10 times longer than the above-ground height. This extensive rooting system enables the plant to maintain higher water potential during drought periods and this confers an advantage on the growth and development of the plant (Wasaya *et al.,* 2018). When plants sense drought, they dynamically alter their root system architecture by changing the root growth, although these characteristics are species-specific (Deak & Malamy, 2005; Den Herder *et al.,* 2010). Root elongation and branching are also reduced during severe drought conditions (Smith & De Smet, 2012).

1.5.2. Drought tolerance-associated mechanisms

Drought tolerance is found when plants are able to maintain their normal function under low leaf water status (Yates *et al.,* 2019). Through the regulation of numerous genes and several metabolic pathways, plants are able to withstand certain levels of physiological activities when subjected to drought conditions (Luo, 2010). Plants usually do this with accumulating both inorganic and organic substances to improve water retention by reducing the osmotic potential (Blum & Tuberosa, 2018).

Phytohormones are essential in the normal growth and development of plants under normal growing conditions and when the plants face environmental stress. They are crucial in the coordination of responses to drought and other abiotic stress (Peleg & Blumwald, 2011). Of all the phytohormones, abscisic acid (ABA) is most closely related to plant drought response (Daszkowska-Golec, 2016). Studies have shown that an increase in the *de novo* synthesis of ABA is triggered when root cells sense water deficit conditions (Sauter *et al.,* 2001).

Abscisic acid has been reported to play an essential role in the regulation of stomatal closure during drought stress, controlling the activities of the guard cells (Tardieu & Davies, 1992). ABA also regulates the expression of many ABA-responsive genes, enhances the synthetic activity of glutamyl-phosphate and regulate the transcription level of calmodulin protein (Rabbani *et al.,* 2003; Mori *et al.,* 2006). ABA has also been reported to improve the synthesis and accumulation of proline by enhancing the activity of pyrroline-5-carboxylate reductase (P5CR) (Verslues & Bray, 2006; Verbruggen & Hermans, 2008).

Drought stress has been reported to affect chlorophyll content, directly and indirectly. Li *et al.* (2006) have shown that chlorophyll content is significantly reduced when reducing soil water content. The chlorophylls Chl *a* and Chl *b* are virtually essential pigments for the conversion of light energy to stored chemical energy. It has been speculated that a reduction in Chl *a* causes a substantial decrease in the total chlorophyll content during drought stress (Kocheva *et al.*, 2005).

Plants are said to be light energy efficient if they are able to maintain higher chlorophyll content during drought stress, thus having increased tolerance to drought (Guo *et al.,* 2009).

Chlorophyll fluorescence has been described in many studies relating to drought tolerance, in many plants, such as cotton, potato, maize, and tomatoes (Schapendonk *et al.,* 1989; Shahenshah and Isoda, 2010; Ni *et al.,* 2015; Yuan *et al.,* 2016). It can be described in terms of the light chlorophyll molecules re-emitted after moving from the excited state to a non-excited state (Lu, 1999). The photosystem (PSII) is very sensitive to both abiotic and biotic stresses. This makes chlorophyll fluorescence an important tool with which to screen for drought-tolerant genotypes that are able to protect photosystem II (PSII) and maintain photochemistry. Chlorophyll fluorescence can be determined using a chlorophyll fluorimeter in a non-destructive way.

Plants can also respond to drought stress by accumulating both organic and inorganic substances; this significantly lowers the osmotic potential and prevents water loss (Turner *et al.,* 2001). The ability of plants to adjust their osmotic potential has been documented in many studies (Gobu *et al.,* 2017). Adjusting osmotic potential during water deficit conditions can enable a plant to delay leaf senescence, improve root growth and prevent death. The ability and the extent to which plants can adjust the level of drought tolerance in many plant species (Sanchez *et al.,* 2012; Silvente *et al.,* 2012). This implies that different plant species (genotypes) might use different tolerance strategies, however several metabolites are conserved among species (Sanchez *et al.,* 2012).

Many organic compounds, such as proline, mannitol, glycine betaine and inositol, have been found to contribute significantly to reducing osmotic potential during drought stress (Szabados *et al.,* 2011). These compounds are referred to as osmoprotectants, which protect the plasma membranes and enzymes during drought stress and help plants re-establish osmotic homeostasis by increasing water potential. The composition and concentration of osmoprotectants vary considerably in stressed plants, depending largely on the species and effect of the external environment (Evers *et al.,* 2010; Lugan *et al.,* 2010).

Proline has been widely studied as an osmoprotectant, and reports are inclined toward the accumulation of proline during drought stress. The structure of proline makes it suitable as an osmoprotectant. Proline has very strong hydration ability, the hydrophilic part binds to water molecules while the hydrophobic parts bind to proteins, and this enables the proteins to access more water while protecting them from denaturing, especially during water deficit (Hoekstra *et al.,* 2001). For example, Jungklang *et al*. (2015) reported an increase in the accumulation of proline when water was withheld for 30 days in *Curcuma alismatifolia* plants. Proline has been

shown to play an important role in protecting protein and cellular redox homeostasis from stress-induced damage (Verbruggen & Hermans, 2008; Szabados & Savoure, 2010). Pyrroline-5-carboxylate reductase (P5CR) might play a central role in proline accumulation in plants that are responsive to stresses. Dudziak *et al*. (2019) showed that the expression level of P5CR genes increased when wheat plants were subjected to drought stress.

Late embryogenesis abundant proteins (LEA) also have important roles in osmotic adjustments in plants. The LEA proteins are a family of low molecular weight proteins and are formed during seed development (Hongbo *et al.,* 2006). These proteins are extremely thermally stable and usually remain in an aqueous state under boiling conditions, because the protein is hyper-hydrophilic. They have been reported to prevent excessive dehydration in plant tissues, protect biological molecules, and bind to inorganic ions to prevent the damage caused by the accumulation of high concentrations of ions in water deficit conditions (Fang & Xiong, 2015). LEA proteins can also bind to nucleic acid and control the expression of genes (Close, 1996). Dehydrins are examples of LEA proteins and the dehydrins gene is strongly expressed during drought conditions (Wahid & Close, 2007).

Aquaporins are membrane channel proteins responsible for water transportation and are ubiquitous in all kingdoms except thermophilic Archaea and intracellular bacteria (Maurel *et al.,* 2008). Aquaporins play a vital role in mediating the passive water transport achieved by an osmotic pressure gradient from within and outside the membranes (Maurel, 1997; Maurel *et al.,* 2008). The maintenance of a cell's osmotic potential during drought stress is a major constraint on the growth and development of the plant. Aquaporins are vital regulators in plant water retention and have been potential targets when developing drought-resistant plants (Park & Campbell, 2015).

Aquaporins are divided into three groups based on their subcellular location: the nodulin-26-like major intrinsic proteins (NIMs), the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs). Alexandersson *et al.* (2005) examined the expression level of 35 aquaporin analogues in Arabidopsis during drought stress and reported that almost all PIP genes were highly expressed; the NIP genes were poorly expressed. Other researchers have reported that most of the PIP genes were the most responsive to drought stress and were down-regulated in leaves (Jang *et al.,* 2004; Rizhsky *et al.,* 2004; Alexandersson *et al.,* 2010). The PIP genes were found to be down-regulated during drought stress in the roots and twigs of olive plants (Secchi *et al.,* 2007).

Antioxidant defence systems are also an integral part of drought tolerance. When plants are subjected to water deficit conditions, the stomatal closure limits the photosynthetic efficiency in the leaves and results in enhanced photorespiration, over-reduction in the photosynthetic electron transport chain and increases in the production of oxidising agents, especially the reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Excess ROS accumulation in the cells during drought stress contributes to the destruction of many cellular components, and damages the membrane phospholipids and fatty acids in a process described as membrane lipid peroxidation (Moller, 2001). Membrane lipid peroxidation leads to increased permeability and ionic leakage, disturbance in the normal cell metabolism, dysfunction in the chlorophyll and even plant death. This means that the ROS is an important signal in plant responses to drought stress (Møller *et al.,* 2007; Gill & Tuteja, 2010).

Antioxidants in the cellular context are molecules that donate hydrogen atoms or electrons to ROS, rendering them harmless. Plants generally have both enzymatic and non-enzymatic anti-oxidation systems. Generally, the non-enzymatic antioxidant defences in the cell are made up of low molecular weight compounds such as carotenoids, glutathione, α-tocopherol and ascorbic acid. The non-enzymatic antioxidant system donates hydrogen atoms or electrons to oxidising agents. The enzymatic antioxidants are proteins that scavenge ROS, usually by electrons provided in the non-enzymatic pathway. Some of the enzymes involved directly in the antioxidant defence mechanism are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and the thiol peroxidases of peroxiredoxins (Sevilla *et al.,* 2015; Hussain *et al.,* 2016)

Under normal conditions, ROS production is neutralised by the activity of the antioxidants, however, during drought stress, the production of ROS exceeds their neutralisation (Figure 3). Understanding the way in which plants respond to drought at the molecular level is essential for developing improved genotypes that are well suited for growth and development in regions that have limited water (Ribaut *et al.,* 2009).

How a plant tolerates drought is a complex quantitative trait, however, which involves multiple metabolic pathways (Khowaja & Price, 2008) and the activation or suppression of many genes. A myriad of genes are involved in the plant response, and tolerance to drought has been identified in some model plants (Guo *et al.,* 2009).

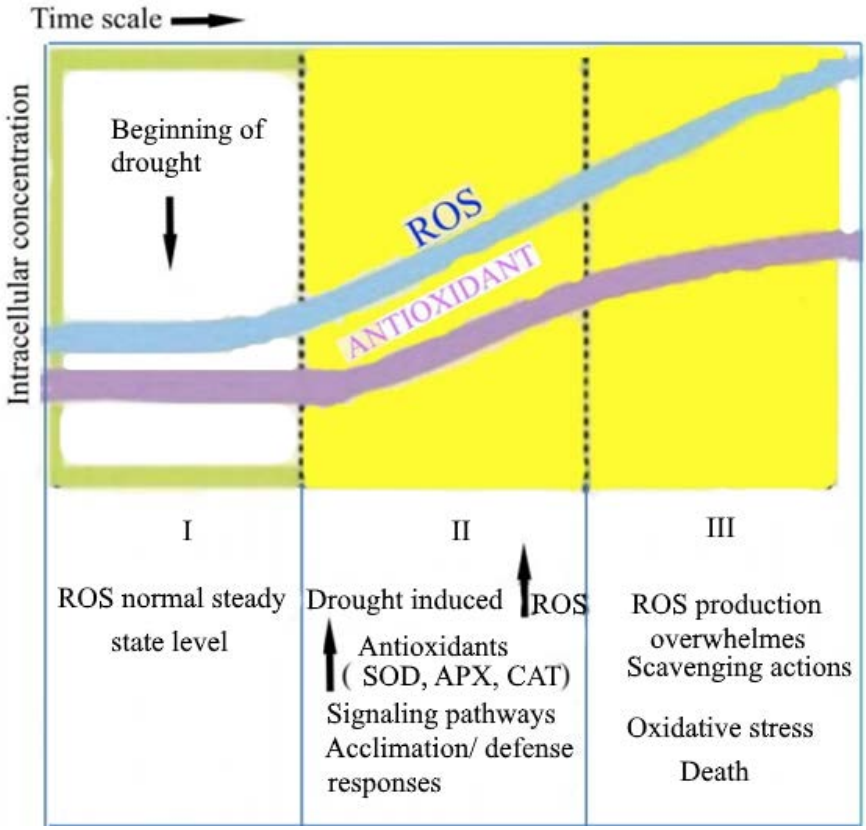


Figure 3. *Proposed model for drought stress response (adapted from Cruz de Carvalho, 2008)*

Some of the genes are involved in anti-oxidation activities needed to produce substrates that reduce the elevated concentration of ROS arising as a result of drought and other abiotic stress (Zhang & Sonnewald, 2017; Zandalinas *et al.*, 2018). The upregulation of antioxidant enzymes has been reported in many studies and serves as an important marker for drought stress (Luna *et al.*, 2005; Sofo *et al.*, 2015; Cao *et al.*, 2017).

1.6. Salinity stress and its impact on plant productivity

Salinity remains one of the factors that limit the productivity of many crops. Soil irrigation combined with poor drainage systems have resulted in the salinisation of more than 45 million hectares (Zhu, 2007; Munns & Tester, 2008). Water used for irrigation often contains magnesium, sodium and calcium. During evaporation, the calcium and magnesium are precipitated to carbonates while the sodium ions become dominant in the soil (Serrano, Culianz-Macia, & Moreno, 1999). Over time, the sodium ions accumulate in the soil and exceed the concentration of other macronutrients in one or two folds.

Sodium chloride is commonly used to melt ice and protect roads during winter periods and has also been found to increase soil salinity (Turner *et al.*, 2013). The sodium ion becomes attached to soil particles, displacing phosphorus and potassium. This often results in increased compactness

and soil density while reducing aeration and drainage. When soil salinity is not managed properly and efficiently, soil sodicity results, which damages the soil structure. When sodium ions occupy the cation exchange complex of clay particles, it causes the soil aggregate to disintegrate, reduce in porosity, and increase in bulk density, and hampers aeration in the soil (Singh & Chatrath, 2001). In addition to the effect of sodium toxicity, plants are also affected to some degree by hypoxia resulting from poor aeration (Tisdale *et al.,* 1993).

Salinity tolerance is affected by environmental factors such as radiation, temperature, pressure deficit vapour, as well as the soil type (Chinnusamy *et al.,* 2005). The salt level in soils fluctuates seasonally and spatially (Yao *et al.,* 2016) For example, the continuous growing of vegetables in the same soil has been found to increase salinisation (Machado & Serralheiro, 2017). Salinity still limits the productivity of many crops. Soil irrigation has resulted in the salinisation of more than 45 million hectares (Munns & Tester, 2008). Plants grown under salinity stress have decreased marketable yields as a result of decreased productivity. Salinity causes nutritional disorder related to calcium deficiency and manifests by increasing blossom-end rot in tomato, pepper fruits, and eggplants, and thus reducing their commercial value (Machado& Serralheiro, 2017).

Salt stress has a negative effect on germination, plant vigour and yield (Munns & Tester, 2008). When plants are exposed to high salinity stress, all major physiological and biochemical processes such as protein synthesis, photosynthesis and energy metabolism are affected (Parida & Das, 2005). Salinity reduces the photosynthetic apparatus by limiting the availability of CO_2, usually by diffusion limitations (Flexas *et al.,* 2007) and a reduction in the chlorophyll content (Delfine *et al.,* 2013; Ashra & Harris, 2013), a reduction in leaf growth and inhibited photosynthesis, leading to a significant loss in yield (Yeo, 2007). In spinach, salinity stress inhibits photosynthesis by limiting the stomatal and mesophyll conductance to CO_2 (Delfine *et al.,* 1998; Di Martino *et al.,* 1999). A reduction in chlorophyll content significantly affects light absorbance (Alvino *et al.,* 2000). In other plants, such as radishes, salinity significantly reduces growth and this reduction is attributed to the limitation in light reception as a result of the tremendous reduction in the leaf area expansion (Marcelis & Van Hooijdonk, 1999).

High soil salinity affects the uptake of water from the roots and also affects cell growth and metabolism in the roots. Highly saline conditions in the soil often lead to structural defects, high root zone pH, oxygen deficiency, impaired root respiration and nutritional imbalances

(Roy *et al.,* 2014). Studies have shown that plants grown on high saline substrates demonstrate salt-specific stress, oxidative stress and osmotic and ionic stress (Muscolo *et al.,* 2013).

High soil salinity often results in hyperosmotic stress, where there is a progressive loss of water from the leaves while the absorption of water by the roots is significantly reduced (Tang *et al.*, 2015). This triggers physiological changes that are detrimental to the development of the plant, including a reduction in photosynthetic activities and an increase in the reactive oxygen species (ROS) produced (Acosta-Motos *et al.*, 2017). The ROS resulting from oxidative stress caused by high salinity substrate damages the cellular components and disrupts important cellular functions. The osmotic effect of salinity stress can be observed at the onset of the salt application; the growth rate declines (Figure 4), and cell expansion and division is inhibited as the exposure progresses (Munns, 2002; Flowers, 2004).

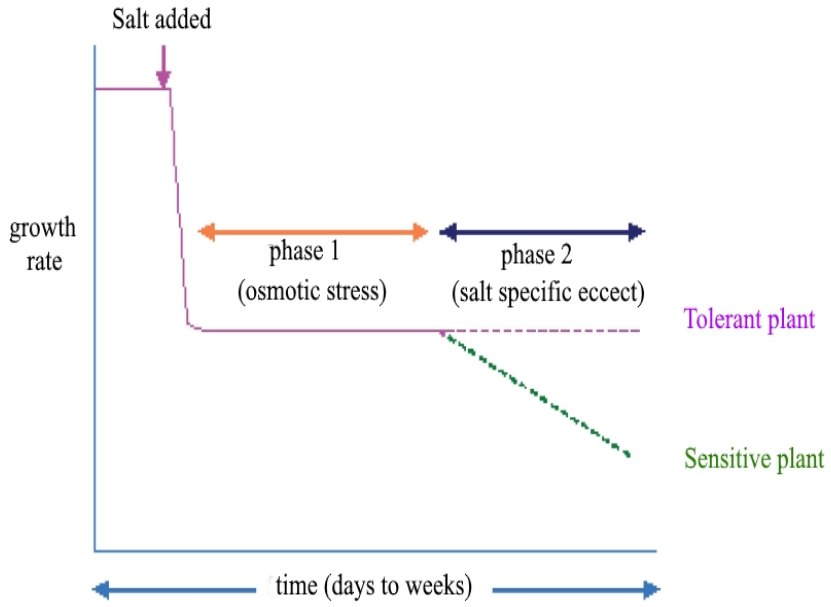


Figure 4. *Two-phase growth response to salinity (Munns et al., 1995)*

Ionic stress is manifested by the accumulation of sodium and chloride ions in a plant's tissues. The uptake of these ions causes a severe ionic imbalance as the high concentration of sodium ions inhibits the uptake of potassium ions (Assaha *et al.*, 2017). The unavailability of potassium ions results in physiological impairment in the growth and development of plants and can result in plant death. Excess sodium and chloride ions have been found to affect or inhibit enzymatic functions in the cells, leading to a reduction in energy production and other physiological changes. When plants are exposed to long-term salinity stress, ionic stress often leads to premature leaf senescence, and hence, a significant reduction in the photosynthetic area available to support adequate growth and development (Cramer & Nowak, 1992).

When plants are subjected to salinity stress, the visual symptoms of plant growth appear progressively. In the early stages, wilting, the yellowing of leaves and stunted growth are visible.

As the stress progresses, the damage manifests as leaf tip burning, the chlorosis of green parts, and necrosis of leaves, and the old leaves display scorching (Shannon & Grieve, 1998).

1.7. Whole-genome duplication (WGD) in plants

WGD, also known as polyploidisation, is a phenomenon by which the whole genome of the cells in an organism is duplicated. It encompasses multiple processes that result in the formation of a polyploid organism, with three or more sets of the base chromosome number (Madlung, 2013). Polyploids are formed as a result of nondisjunction during meiosis, enabling the cells to have more than two homologous sets of chromosomes (Chen, 2010).

Polyploidy is common in species of vascular plants and angiosperms (Wendel, 2000; Moghe & Shiu, 2014). Many researchers have seen polyploidisation as advantageous for changing species morphology and physiology, enabling such species to adapt to their environment and gain evolutionary success. WGD is one of the most critical alterations in a genome (Ramsey & Ramsey, 2014).

WGD can either be autopolyploidisation or allopolyploidisation (Chen, 2010; Marfil *et al.*, 2018). Autopolyploidy occurs when a cell has more than two sets of chromosomes from the same parental species, and allopolyploidy occurs when a cell has more than two sets of chromosomes, and the additional set of chromosomes come from different species. Polyploids can occur naturally or can be induced from diploid cells in order to obtain lines demonstrating new agronomical characteristics through the application of anti-mitotic substances that block the cell cycle.

1.8. Anti-tubulin drugs

The cell cycle can be divided into four major steps: the S-phase in which DNA replication occurs, preceded by the G1 phase and G2 phase, and finally mitosis (the M-phase). The distribution of genetic materials equally into daughter cells occurs after the DNA replication process during cell division.

The mitotic spindle plays an important role in the accurate separation of sister chromatids into two daughter cells during cell division. The mitotic spindle is made up of a self-organising bipolar microtubule that harnesses the energy from the hydrolysis of GTP (Hoyt & Geiser, 1996). Microtubules are made up of tubulin, which is a dynamic polymer. The major components of the microtubule are the α-tubulin and β-tubulin polypeptide.

The principle use of anti-tubulin drugs is to inhibit microtubule polymerisation, usually during the metaphase/anaphase stages of the M-phase. Anti-tubulin drugs have been used in several studies to study microtubules (Khosravi *et al.,* 2008; Amiri *et al.,* 2010). Anti-tubulin drugs such as oryzalin, colchicine, trifluralin and amiprophos-methyl (APM) have been used to arrest mitosis at the metaphase stage by inhibiting microtubule polymerisation and ultimately inhibiting spindle formation (Kitamura *et al.,* 2009).

Studies have shown more frequent use of colchicine than amiprophos methyl, oryzalin, trifluralin and nitrous oxide, and this could be attributed to the simplicity of replicating the protocol (Jaskani *et al.,* 2005; Madon *et al.,* 2005; Filipe Almendagna Rodrigues, 2011; Tamayo-Ordóñez *et al.,* 2016). Anti-tubulin drugs such as oryzalin have been found to have a higher affinity for the tubulin-binding sites than colchicine, and therefore chromosome doubling can be achieved at a very low concentration compared to colchicine (Ślusarkiewicz-Jarzina *et al.,* 2017).

Chromosome doubling in ryegrasses is a way to broaden the germplasm, and it is therefore important to develop an easy protocol to achieve optimal results.

1.9. Gene expression pattern in polyploids

Polyploid plants have been found to show better resistance to both biotic and abiotic stress including drought and salinity stress. These resistances enable polyploids to be better adapted to a wider ecological region (Blanc & Wolfe, 2004).

The expression of most genes has been found to be directly proportional to the ploidy level; that is, the expression of genes increases with ploidy. Some genes have been found to be less expressed in tetraploid than their diploid counterpart, however (Guo *et al.,* 1996). Comparing the proteomes of diploid and the polyploids of *Brassica* species showed there was no significant difference either qualitatively and quantitatively, although the analysis of mRNA is more sensitive than that of proteins, which means that changes in the low abundance protein may have been missed (Albertin *et al.,* 2006). Doyle *et al.* (2008) studied the differential gene expression patterns of diploids and polyploids to identify the effect of natural and synthetic polyploids resistance to abiotic stress. Similarly, the molecular basis for evolutionary advantage has been associated with gene expression changes in resynthesised polyploids in *Arabidopsis* (Wang *et al., 2006)* and in *Gossypium* (Flagel *et al.,* 2008). Polyploidy has been found to give rise to many gene expression changes and to new allelic variants (Feldman & Levy, 2009) in addition to causing the expansion of gene families (Veron *et al.,* 2007).

Many homologous genes in newly formed polyploids may be redundant if they have a similar sequence, or one of the genes might be silenced (Wendel, 2000), however, the similarity of genes is not always perfect in polyploids as some genes differ slightly in sequence and mode of regulation. This, in turn, can result in differences in the functions that affect the quantity, time or place of appearance of some metabolite or binding factor (Wendel, 2000).

This study provides useful information that may explain how plants with different ploidy levels respond to abiotic stress. Understanding the differential expression pattern of genes involved in drought response provides insight into how cytotypes respond to water shortage at the molecular level. This study may eventually result in the development of drought resistant lines of Westerwolths ryegrass that produce higher yields in target saline and drought environments.

2. MATERIALS AND METHODS

2.1. Tetraploid induction from diploid cultivars of Westerwolths ryegrass

This study was performed at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, Laboratory of Genetics and Physiology. Ten different diploid cultivars of Westerwolths ryegrass were used (Table 1).

Table 1. *Cultivars and induced tetraploid lines used in the field trial (F), salinity stress (S), and drought stress trial in a controlled environment (CE)*

Name	Ploidy	Origin	Trial	Induced tetraploid name	Trial
Druva	Diploid	Institute of Agricultural Resources and Economics (AREI)	F, S and CE	Druva-4x	F, S and CE
Varpė	Diploid	Institute of Agriculture, LAMMC	F, S and CE	Varpė-4x	F, S and CE
Magloire	Diploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	Magloire-4x	F, S and CE
Top speed	Diploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	Top speed-4x	F, S and CE
Grazer	Diploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	Grazer-4x	F, S and CE
Surrey nova	Diploid	Dansk Landbrugs Froselskab (DLF)	S, CE	Surrey nova-4x	S, CE
Prompt	Diploid	Dansk Landbrugs Froselskab (DLF)	S, CE	Prompt-4x	S, CE
Shoot	Diploid	Dansk Landbrugs Froselskab (DLF)	S, CE	Shoot-4x	S, CE
Weldra	Diploid	Advanta Seeds BV	-	Weldra-4x	-
Aramo	Diploid	Dansk Landbrugs Froselskab (DLF)	-	Aramo-4x	-
Wesley	Tetraploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	-	
Avance	Tetraploid	Dansk Landbrugs Froselskab (DLF)	F	-	
Rapid	Tetraploid	Federal Williams Research Centre of Forage Production and Agroecology (FWRC FPA)	F	-	

Caremo	Tetraploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	-
Peleton	Tetraploid	Dansk Landbrugs Froselskab (DLF)	F, S and CE	-

The method described by Pašakinskienė (2000) was adopted for the sterilisation of seeds and tetraploid induction. Seeds were surfaced sterilised and the embryos were excised from the seeds. Sterilised embryos were sprouted in Petri dishes containing Gamborg B5 (Duchefa Biochemie) medium for 3-5 days at a temperature of 24°C. The coleoptiles were allowed to grow up to 0.5 cm long and then transferred to a 4°C refrigerator for two days to pause the process of mitosis. Prior to the treatment of plants with colchicine and amiprophos methyl (APM), the plants were transferred to the growth chamber at 28°C for one hour. The concentrations and durations of the inhibitors are shown in Table 2.

Table 2. *Treatment used for tetraploid induction in Lolium multiflorum spp. multiflorum*

Mitosis inhibitors	Concentration	Duration
Colchicine	10 mM	3 or 4 h
Colchicine	8 mM	3 or 4 h
Amiprophos methyl	0.1 mM	4 h
Amiprophos methyl	0.05 mM	4 h
Amiprophos methyl	0.04 mM	4 h
Amiprophos methyl	0.03 mM	3 h
Amiprophos methyl	0.02 mM	3 h
Amiprophos methyl	0.015 mM	3 h

The ploidy levels of the survived plants were checked using a Partec PA (Partec Gmbh, Germany) flow cytometer. Root tip squash technique for counting the chromosome number was used to verify the ploidy levels of 15 randomly selected plants.

2.2. Field trials to evaluate the performance of diploid, induced tetraploids and tetraploid cultivars

The experiment was carried out in the fields of the Institute of Agriculture, Lithuania Research Centre for Agriculture and Forestry (55°40’ N, 23°87’ E) during the 2017 and 2018 growing seasons. The field experiment was established in three replicates in a randomised complete block design on May 25th in 2017 and May 9th in 2018, and each line/cultivar was represented by 20 plants per replicate. The soil of the experimental fields in the Grass Breeding Department is

Endocalcari – Epihypogleyic Cambisols (CMg-p-w-can), characterised by a homogeneous texture, pH_{KCl} 7.2, humus content 1.74%, available P_2O_5 175 mg kg^{-1} and K_2O 157 mg kg^{-1}. Fertilisers were applied before sowing $N_{30}P_{50}K_{70}$ and N_{45}, and after cuts.

Plant height (cm), flag leaf area (cm^2) and inflorescence length (cm) were measured when the heading stage was completed (BBCH 59). Ten plants per replicate, three flag leaves and inflorescences per plant were measured using image processing program ImageJ. Infection with *Puccinia coronata* was visually scored. Fresh plant biomass was collected after regrowth from the first cut, dried and weighed to obtain the dry matter yield (DMY) from each cultivar and from the induced tetraploid lines.

The 2017 and 2018 growing seasons in Lithuania had different climatic conditions, especially in terms of the amount of rainfall and temperature, as shown in Figure 5. The summer of 2017 was rainy and cool, and the mean temperatures reached 20°C only at the end of July, whereas 2018 was much warmer, with less precipitation and lower air humidity; hence plants grown during 2018 were exposed to drought periods compared to the preceding growing season.

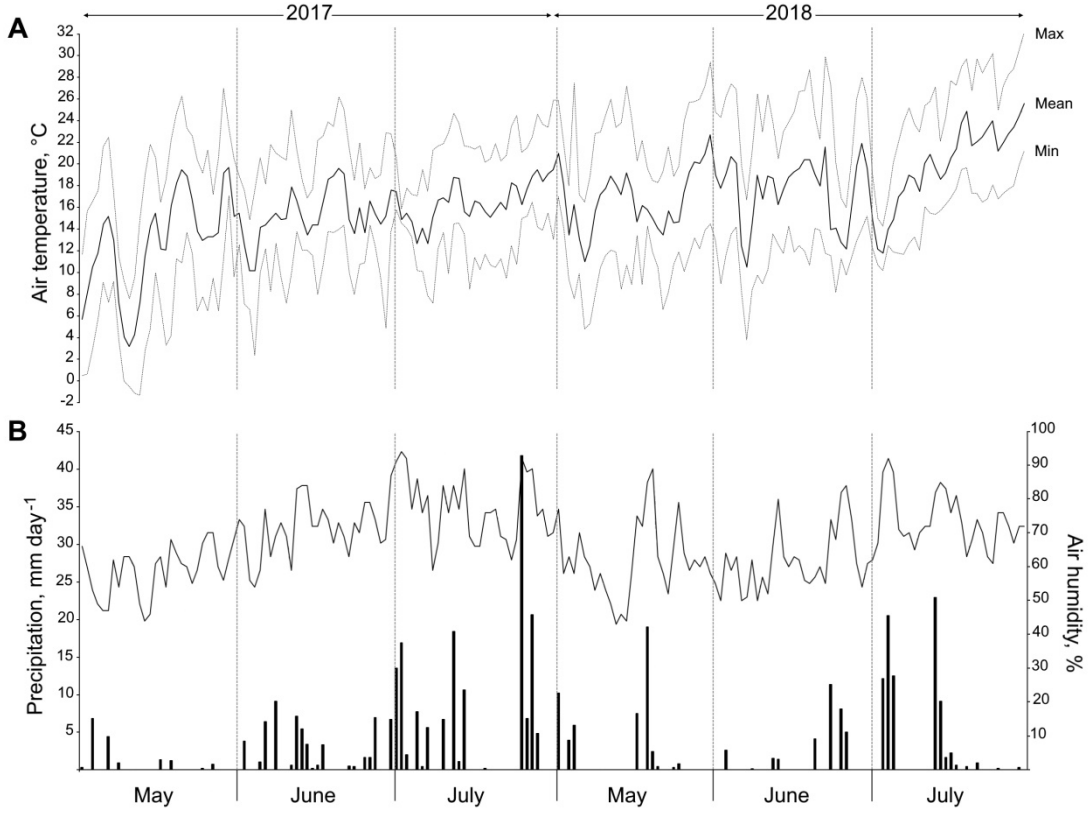


Figure 5. *Meteorological conditions during 2017 and 2018, Akademija, Lithuania. (A) Average, maximum and minimum air temperature, °C; (B) average precipitation, mm d−1 and air humidity, %*

2.3. Mild drought simulation in the controlled environment

Eight diploid cultivars, their respective induced tetraploid lines and three tetraploid cultivars were used in the drought simulation experiment. The seeds were germinated on a filter paper and seedlings were allowed to grow for seven days before transplanting them to the round plastic pots (diameter 9 cm, height 8 cm) filled with 350 g sandy soil (54% compost, 32% sand, 14% peat), five plants per pot, four pots per line/cultivar. The pots were placed at random in the phytotron, set to 24℃ during the day and 18℃ at night, a 16/8 hour photo-period and relative humidity of 60%. The plants were watered for two weeks and excess water was drained from holes at the base of the pots. Seven days before the inducing mild drought, 100 ml of water was added to each pot at the same time of the day to enable the plants to carry out their normal physiological functions. This was done to avoid excess water in the soil while preparing for drought initiation.

To investigate the effect of mild drought, 10 ml of water was added to each pot daily for five days. Five new unfolding leaves were marked at the nodes on the first day and the leaf elongation was recorded daily at the same time. Leaf wilting was also observed at the end of the mild drought simulation and scored from no wilting to severely wilted (1 – no sign of wilting, 6 – severely wilted). The experiment was carried out in three replicates. Severe drought commenced immediately after mild drought. Water was completely withheld for five days. The survival rate was determined

2.3.1. Determination of the relative water content and chlorophyll fluorescence

The relative water content (RWC) was determined at the end of the drought treatment. Leaf samples were collected from each cultivar/line and weighed immediately to obtain the fresh weight (FW). The leaf samples were transferred to plastic sacks containing water and left for six hours before obtaining the turgid weight (TW). Finally, the leaf samples were blotted dry and placed in an oven at 70°C for 72 hours and weighed to obtain the dry weight (DW). The RWC was calculated using the formula:

RWC % = 100[(FW - DW) / (TW - DW)] (Smart & Bingham 1974).

The chlorophyll fluorescence (Fv/Fm) was measured using a chlorophyll fluorometer OS30p+ (Opti-Sciences Inc. USA).

2.3.2. Antiradical activity measurement

The research was carried out in the laboratory of Biochemistry and Technology, Institute of Horticulture, Lithuanian Research Center for Agriculture and Forestry. 2,2-diphenyl-1-

picrylhydrazyl (DDPH) free radical scavenging activity was determined by modifying the method described in Brand-Williams *et al*. (1995). The leaf samples were collected after mild drought and dried in an oven at 40°C for four days, homogenised and 0.5 g of the homogenised leaves was suspended in 70% methanol. The extraction was done in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH and Co. KG, Germany) for 60 min at 50°C and 480w. 2 ml of DDPH solution in 70% v/v methanol was mixed in 2 μL methanol extract. The reduction in absorbance at 515 nm was measured and expressed as Trolox equivalent antioxidant capacity.

2.3.3. Determination of total phenolic content

Spectrophotometric measurements were carried out with a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, USA). The total phenolic content (mg GAE/100 g DW) in the methanol (99.0%, v/v) of Westerwolths ryegrass leaves was determined by the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE).

2.4. Evaluation of seed germination parameters during salinity stress

Fifteen seeds of each cultivar and induced tetraploid line were placed on three layers of filter paper in Petri dishes in three replicates. The filter papers were moistened with either distilled water or different concentrations of sodium chloride (NaCl) solution. The salinity concentrations ranged from 120 to 200 mM. The germination was recorded daily for 10 days. The experiment was repeated three times. The germination percentage (GP), germination index (GI), mean germination time (MGT) and the time to reach 50% of the germination of all the seeds (T50) values were calculated as described in Coolbear *et al*. (1984) and Kader (2005), as modified by Farooq *et al*. (2005):

$GP = 100 (x/n)$,

where x is the total number of germinated seeds, n – the total number of seeds;

$GI = (10 \times n1) + (9 \times n2) + \cdot \cdot \cdot + (1 \times n10)$,

where n1, n2 . . . n10 represent the germinated seed on the first, second and subsequently days until the 10^{th} day; 10, 9 . . . 1 are weights given to the number of germinated seeds on the first, second and subsequent days until the 10^{th} day, respectively;

$MGT = \sum nt / \sum n$,

where t (days) represents the time from the beginning of germination test, n – the number of germinated seeds at time t.

$$T50 = \frac{ti + [\{(N / 2) - ni\}(ti - tj)]}{ni - nj},$$

where N represents the final number of germination, n_i and n_j are cumulative number of seeds germinated by adjacent counts at times t_i and t_j when $n_i < N/2 < n_j$.

2.5. Evaluation of salinity stress on Westerwolths ryegrass seedlings

Seeds from diploid, tetraploid and induced tetraploid lines and cultivars of Westerwolths ryegrass were germinated on 50:50 perlite: vermiculite mix (vol.) substrate in round plastic pots (diameter 9 cm, height 8 cm). The plantlets were allowed to develop for three weeks at 25 ± 2°C with a 16/8 h light/dark photoperiod before inducing salinity stress. The plantlets were treated with 500 mM NaCl for ten days. New unfolding leaves were marked at the nodes on the first day in five different plants in separate pots, and the leaf elongation was recorded on a daily basis at exactly the same time. The treatment was replicated three times.

The RWC, antiradical activity and phenolic content of the plants were determined after salinity stress, as described in the previous sections.

2.6. Gene expression analysis

2.6.1. Plant preparation

Seeds from Varpė and Magloire and their respective induced tetraploids were germinated on filter papers and transplanted to an equal volume of sandy soil in round plastic pots (four plants per pot and 48 pots per line/cultivar. The plants were grown for three weeks prior to subjection to drought stress. Three time-points (one day after the last watering, three days after the last watering and five days after the last watering) were selected to check for changes in the expression level of the chosen candidate genes (Table 3). The relative water content was determined by methods described in Section 2.3.1.

2.6.2. RNA preparation, cDNA synthesis and qPCR

Total RNA was extracted from diploid and induced tetraploid leaves of both stressed and unstressed plants (Magloire and Varpė) using the GeneJet Plant RNA purification minikit (Thermo Fisher Scientific, Lithuania) in accordance with the manufacturer's protocol. To remove genomic DNA contaminants, a digestion by DNase (Thermo Fisher Scientific, Lithuania) was performed on the RNA samples. RNA quantification was done using NanoDrop 2000 (Thermo Fisher Scientific, USA) spectrophotometer, RNA quality was checked by performing electrophoresis in 1% agarose gel. The first-strand cDNAs were synthesized from 1 µg of total

purified RNA using the RevertAid First-strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) following the manufacturer's instructions. cDNA samples were diluted 15-fold prior qPCR analysis. Amplification and detection were performed using PowerUp SYBR Green Master Mix (Applied Biosystems, UK) in 7500 Fast Real Time PCR System (Applied Biosystems, USA). Gene expression analyses were performed using 4 µl diluted cDNA sample in 10 µl reaction volume and 0.5 µl of 5 mM gene-specific primers. Four technical and 3 biological replicates were used per each time point.

Table 3. *Primers for candidate and reference genes selected for real-time PCR*

Gene	Gene function	Forward primer 5' ⟶ 3'	Reverse primer 5' ⟶ 3'
Dh3	Dehydrin part of the late embryogenesis abundant proteins	CGGCACCTATGGACAGCA	CCACAGAGGACATGAACCC
Cu/Zn SOD	copper/zinc superoxide dismutase	ACCACCGTGACAGGAAGC	AACAACATTGATGGGAGCA
GPX	glutathione peroxidase	AAGGCCGAGTATCCGATTT	GCGAGCAGCTTCTTGAGG
eEF1A	Eukaryotic elongation factor 1 alpha	CCGTTTTGTCGAGTTTGGT	AGCAACTGTAACCGAACATAGC
CAT	Catalase	AGCTCTTCGTGCAGGTCATC	CAGCATCTTGTCGTCGGAGT
POD	Guaiacol peroxidase	CTCTACAACGAGACCAACATCAA	GTAGACGTTGTCGAAGGAGTACG
HUB1	subfamily of ubiquitin-like post-translational modifiers	CCATCGGCGACCTCAAGAAG	GAGGGTGATGTGGTCCTTGTA
LpP5CR	Pyrroline-5-carboxylate reductase	GGCCTTGCATCTCAGACAGT	TATCAGTGTCCCGCGGAATG
APX	Ascorbate peroxidase	CCTGAAAGGTCTGGGTTTGA	TCCTTGGCATAAAGGTCCAC
TBP	26S proteasome regulatory subunit 6A homolog	TGCTTAGTTCCCCTAAGATAGTGA	CTGAGACCAAACACGATTTCA
YT521	YT521-B-like family protein	TGTAGCTTGATCGCATACCC	ACTCCCTGGTAGCCACCTT

2.7. Statistical analyses

Pairwise t-test, analysis of variance (ANOVA) with post hoc Duncan multiple range tests were calculated with using SAS (Statistical Analysis System). Pearson's correlation coefficients were used to investigate relationships between selected variables. Mean±SE (standard error of mean) were used to describe the variability of measurements.

3. RESULTS AND DISCUSSION

3.1. Tetraploid induction

Chromosome doubling was achieved to varying degrees using different mitosis inhibitors, however, the survival and induction rate depended on the affinity and toxicity of the inhibitors. In our experiments, different tetraploid induction rates were obtained using different concentrations and exposure times for the mitosis inhibitors. Results from the 'Druva' and 'Grazer' cultivars of Westerwolths ryegrass (Table 4) showed that both colchicine and APM were capable of inducing tetraploids from diploid cultivars. APM induced tetraploids from diploid cultivars at a concentration much lower than that of colchicine, although the efficiency is lower. The highest colchicine tetraploid induction was achieved using a concentration of 10 mM with an exposure time of three hours. APM in low concentrations still appeared to be highly toxic to the plants. An APM concentration of 0.05 mM with a four-hour exposure resulted in 10% efficiency of the treated plants but reducing the exposure time to three hours and the concentration to 0.015 mM further lowered the treatment efficiency to 6.7% in the 'Druva' cultivar.

Table 4. *Comparison of the survival and induction of tetraploid rate in two cultivars using amiprophos methyl and colchicine at different concentrations and exposure time*

Mitosis inhibitors in various conc. time interval	Efficiency, %	
	Druva	Grazer
10mM colchicine, 4 h	17.4	17.7
10mM colchicine, 3 h	63.1	44.1
8mM colchicine, 3 h	15.3	19.5
8mM colchicine, 4 h	37.3	35.3
0.1 mM amiprophos methyl, 4 h	0	0
0.05 mM amiprophos methyl, 4 h	10.0	16.0
0.04 mM amiprophos methyl, 4 h	10.0	18.0
0.03 mM amiprophos methyl, 3 h	7.6	6.2
0.02 mM amiprophos methyl, 3 h	7.0	5.8
0.015 mM amiprophos methyl, 3 h	6.7	-

The survival rate with colchicine and maximum tetraploid induction rates appeared to be better than with amiprophos methyl. A similar experiment to compare and evaluate the efficiency of colchicine and amiprophos methyl on double haploid production of onions was undertaken by Foschi *et al.* (2013). They found that colchicine was more efficient in doubling chromosomes than amiprophos methyl at the same exposure time, although a higher concentration of colchicine was necessary to induce polyploidy than amiprophos methyl.

Weiler *et al.* (2014) found that treating seeds with mitosis inhibitors was more effective than seedling treatment in *Paspalum notatum*. They observed that treating seedlings with a colchicine concentration of 0.1% and higher and for a longer duration (18–24 h) was highly toxic to the plants. Pereira *et al.* (2014) showed that tetraploid induction in *Lolium multiflorum* using 15–20 day old seedlings were not possible at a concentration of 12.5 mM colchicine for 24 hours. The optimal concentration resulted in a 32% survival rate and 27% induction rate when the treatment was composed of 1% DMSO in solution with 12.5 mM colchicine for 24 h (Pereira *et al.*, 2014). Our experiment, however, showed that a high rate of tetraploid induction with lower concentrations of colchicine and shorter exposure time was achievable. The optimal concentration for tetraploid induction in Westerwolths ryegrass (Grazer) was achieved using colchicine treatment with a concentration of 10 mM for 3 h, which resulted in an induction efficiency of 44.1%.

Many factors, such as the concentration of colchicine, plant genotype, the exposure time and the treated seedling organ, have been found to determine the efficiency of colchicine in inducing polyploids in *Rosa* species (Khosravi *et al.*, 2008). Based on the results reported in Table 3.1, two combinations of colchicine concentration and exposure time (10 mM colchicine, 3 h and 8 mM colchicine, 4 h) were chosen to induce tetraploids in eight different cultivars of Westerwolths ryegrass. The differences in the induction efficiency among cultivars are shown in Figure 6.

Induction of tetraploids from diploid cultivars of Westerwolths ryegrass can be achieved using both colchicine and APM, however, the efficiency of the mitosis inhibitors depends on the optimal concentration, exposure time, affinity and toxicity of the inhibitors. The genotypic difference in the cultivars also appears to impact the efficiency of colchicine in this study. A colchicine concentration of 10 mM and 8 mM with an exposure time of 3 and 4 hours respectively was found to be most efficient in inducing tetraploids from diploid cultivars of Westerwolths ryegrass.

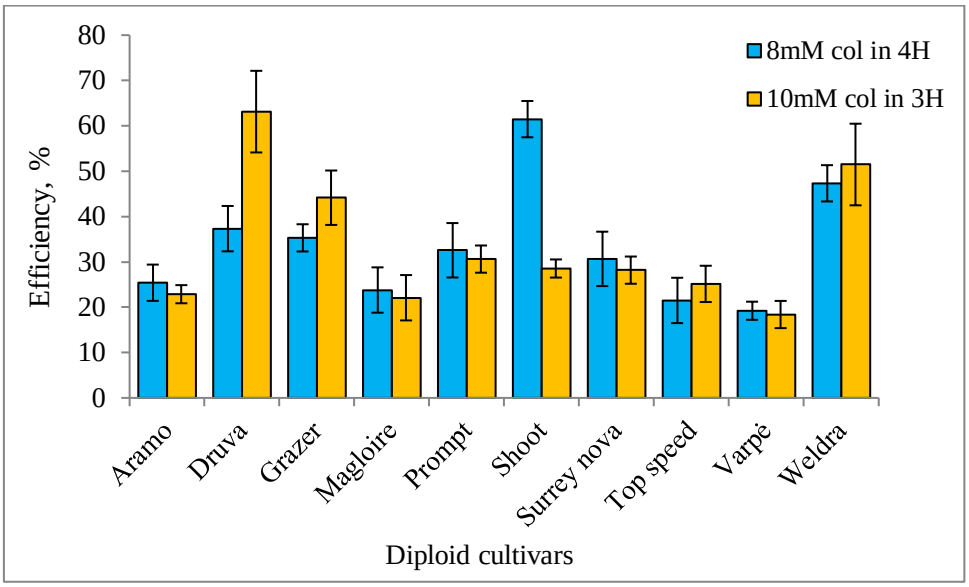


Figure 6. *The efficiency of colchicine in inducing tetraploids in 10 diploid cultivars of Westerwolths ryegrass at different concentrations and exposure time. The error bar represents the standard error of the mean*

The summary

Developing an efficient protocol for induce tetraploid lines from diploid cultivars of Westerwolths ryegrass is critical. Different concentrations of mitosis inhibitors were tested on two diploid cultivars to determine the optimal concentration and exposure time. Colchicine concentrations of 10 mM and 8 mM with exposure times of 3 and 4 hours respectively were found to be the most efficient in inducing tetraploids from diploid cultivars of Westerwolths ryegrass.

3.2. Physiological and morphological response to mild drought

The phenolic compounds and antiradical activity in response to mild drought were determined in both diploids and tetraploids. The results showed that the induced tetraploids produced significantly more phenolic compounds and also had more significant antiradical activity in response to mild drought ($p \leq 0.05$) than their diploid progenitors, as shown in Figure 7.

Drought generally increases the production of reactive oxygen species (ROS) in plants, and if cells are poorly protected this ROS could damage membrane lipids, protein and also DNA molecules, leading to cell death. Redox homeostasis occurs when there is equilibrium in the production and scavenging of ROS. Aghaei *et al.* (2009) suggested that the increase in stress tolerance correlates to increased antiradical activity. Our results showed that the induced tetraploids had significantly more antiradical activities than their diploid progenitors. These results are similar to the reports from Meng *et al.* (2011), where an increase in antiradical activities

resulted in increased stress tolerance in auto-induced tetraploid of turnips compared to their diploid counterparts.

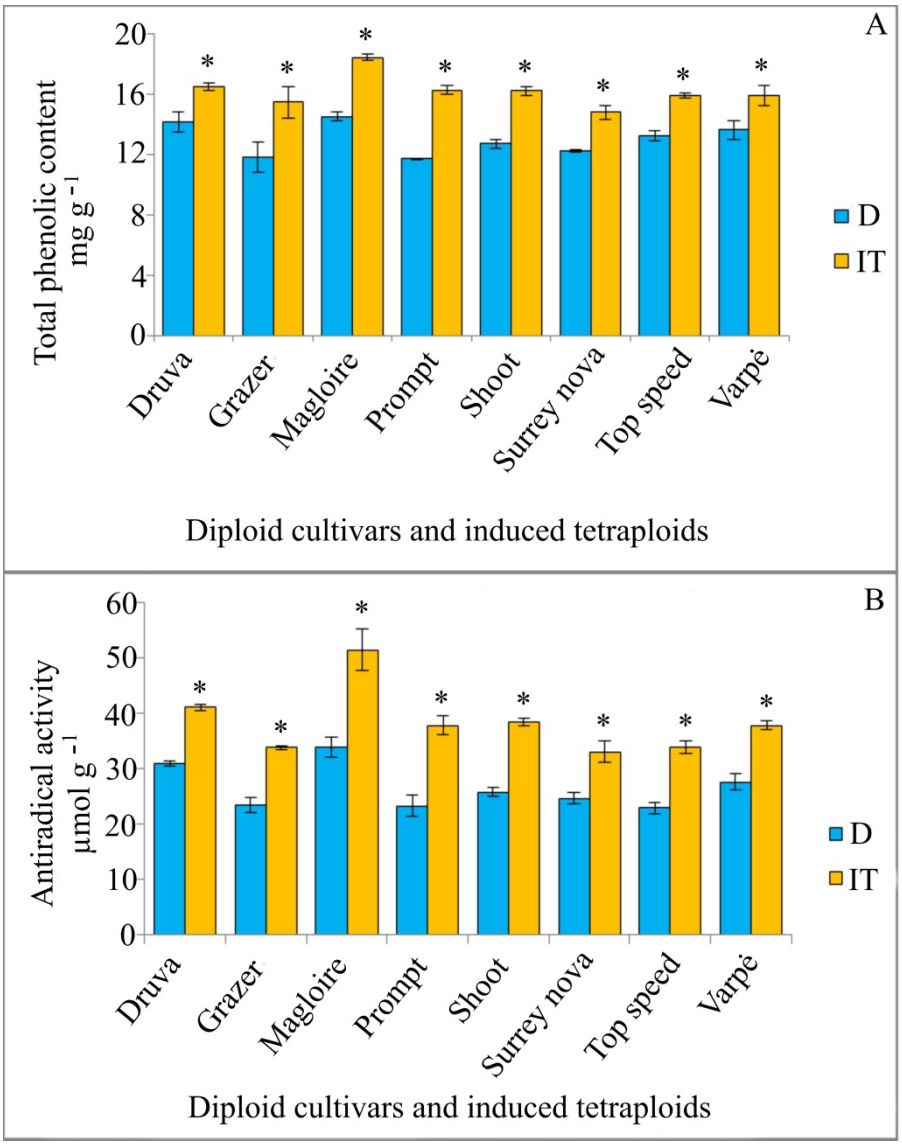


Figure 7. *Total phenolic content (A) and antiradical activities (B) of diploids (D), and induced tetraploid lines (IT) after five days mild drought. Data shown as mean ± standard error of four replicates.*

** represents significant differences $p \leq 0.05$*

Phenolics are secondary metabolites that influence different physiological processes related to growth and development (Tanase *et al.,* 2019). The production of secondary metabolites is often triggered by drought stress, and studies have reported that increases in the production of phenolic compounds such as quercetin and rutin have contributed to tolerance to drought stress in *H. brasiliense* (Abreu & Mazzafera, 2005). In our study, the increase in the antiradical activity and phenolic compounds in the induced tetraploid lines compared to their parental diploids could play an important role in long term exposure to drought, as the induced tetraploid lines could be at an advantage over their diploid progenitors.

Table 5. *Comparison of leaf length (LL), leaf relative water content (LWC) chlorophyll fluorescence (Fv/Fm) and wilting score of diploid cultivars, induced tetraploid lines and tetraploid cultivars after the mild drought simulation*

Line/cultivar	LL, cm	Control LL, cm	Reduction in LL, %	RWC, %	Fv/Fm	Wilting score
Magloire 2x	8.8 ± 0.15	13 ± 0.24	32 ± 0.00	71.8 ± 0.82	0.51 ± 0.03	5
Magloire-4x	9.8 ± 0.31*	15 ± 0.29*	35 ± 0.02	79.6 ± 0.92*	0.63 ± 0.00*	2
Surrey-nova 2x	9.0 ± 1.22	14.3 ± 0.29	37 ± 0.08	79.9 ± 0.38	0.59 ± 0.03	3
Surrey-nova-4x	8.8 ± 0.70	14.4 ± 0.37	39 ± 0.04	80.8 ± 0.81	0.65 ± 0.01	5
Varpė 2x	8.8 ± 1.10	12.3 ± 0.49	29 ± 0.07	80.5 ± 0.30	0.61 ± 0.03	4
Varpė-4x	7.4 ± 0.90	14.2 ± 0.28*	48 ± 0.05*	79.5 ± 0.48	0.62 ± 0.03	3
Grazer 2x	7.8 ± 0.24	11.2 ± 0.32	31 ± 0.04	79.5 ± 0.47*	0.65 ± 0.00	3
Grazer-4x	8.8 ± 0.19*	14.7 ± 0.37*	40 ± 0.04*	73.6 ± 1.11	0.62 ± 0.01	6
Shoot 2x	11.3 ± 0.11*	13.2 ± 0.17	15 ± 0.01	79.1 ± 0.44	0.51 ± 0.01	3
Shoot-4x	9.9 ± 0.22	13.4 ± 0.11	26 ± 0.01*	84.3 ± 0.00*	0.60 ± 0.02*	2
Prompt2x	8.9 ± 0.32	12.6 ± 0.16	29 ± 0.02	78.6 ± 0.84*	0.66 ± 0.01*	3
Prompt-4x	10.2 ± 0.45	13.8 ± 0.32*	26 ± 0.02	73.3 ± 0.96	0.51 ± 0.01	6
Top speed 2x	8.3± 0.21	11.7 ± 0.22	31 ± 0.00	80.5 ± 0.96	0.65 ± 0.01*	4
Top speed-4x	8.1± 0.20	13.7 ± 0.28*	42 ± 0.00*	78.1 ± 0.96	0.53 ± 0.02	3
Druva 2x	9.2± 0.18	11.7 ± 0.35	23 ± 0.01	75.7 ± 0.84	0.64 ± 0.02	3
Druva-4x	7.6± 0.62	13.4 ± 0.28*	44 ± 0.03*	77.2 ± 0.93	0.64 ± 0.01	5
Caremo 4x	10.0± 0.17	14.8 ± 0.19	32 ± 0.02	82.9 ± 0.00	0.59 ± 0.05	3
Wesley 4x	10.2± 0.10	13.6 ± 0.22	25 ± 0.02	81.9 ± 1.28	0.58 ± 0.00	3
Peleton 4x	11.0± 0.24	14.4 ± 0.43	24 ± 0.01	81.0 ± 0.96	0.67 ± 0.01	2

The means followed by * between diploids and corresponding induced tetraploids are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Plant tolerance to drought occurs via many mechanisms, including dehydration avoidance and dehydration tolerance (Fang & Xiong, 2015). The results from mild drought simulation showed

that the cultivars demonstrated signs of dehydration avoidance as a first response to drought by reducing their leaf growth

We studied in detail the leaf elongation of plants grown under control and stress conditions and evaluated the percentage decrease in leaf elongation. Five induced tetraploid lines had a significant reduction ($p \leq 0.05$) in leaf growth compared to their diploid counterparts (Table 5). This reduction in leaf elongation correlates with the total phenolic content in the leaves ($r = 0.45$, $p \leq 0.01$). The response to drought stress involves a cascade of reactions involving many genes at the molecular level, however, duplication of the genetic materials seems to have an advantage over the diploid in the first response to drought in Westerwolths ryegrass.

Plants respond to water stress by complex mechanism inducing various physiological, morphological, biochemical and molecular changes. These responses are highly varied among plant species and also between cytotypes (Aslam *et al.,* 2015). Leaf chlorophyll fluorescence reflects the integrity of photosynthetic apparatus or photochemical efficiency of the photosystem II system in the light reaction of photosynthesis (Kaiser, 1987). Studies have used chlorophyll fluorescence and leaf water content to evaluate the drought response different grass species (Merewitz *et al.*, 2011; Jonavičienė *et al.,* 2012, 2014; Shukla *et al.*, 2015). In this study, variations were found in chlorophyll fluorescence across cultivars and induced lines. Variations were also observed in the relative water content of the diploid and their corresponding induced tetraploid lines.

We correlated the morphological and physiological parameters (reduction in leaf elongation, relative water content, chlorophyll fluorescence, antiradical activity and phenolic content) at the end of mild drought simulation and found no strong correlation among these parameters (Table 6) except a medium positive correlation between the reduction in leaf elongation and phenolic contents and strong positive correlation between the antiradical activity and phenolic content ($r = 0.75$, $p \leq 0.01$). Large group of phenolic compounds have antioxidant properties (Rani *et al.,* 2018) and this could explain why the phenolic contents significantly correlated positively with antiradical activity.

Table 6. *Correlation coefficients matrix among the physiological traits in response to mild drought*

LL reduction	0.28**	0.45**	-0.09**	-0.19**	1
RWC	0.14**	0.17**	0.17**	1	-0.19**
Fv/Fm	0	-0.06**	1	0.17**	-0.09**
Phenolic content	0.75**	1	-0.06**	0.17**	0.45**
Antiradical activity	1	0.75**	0	0.14**	0.28**
	Antiradical activity	Phenolic content	Fv/Fm	RWC	LL reduction

Wilting is a physiological and morphological response to drought stress that occurs due to the loss of cell turgor pressure, resulting in the drooping of leaves (Tavakol & Pakniyat, 2007). Wilting under drought stress is common phenomenon and this visual cue is important for assessing drought tolerant plants. As a result of the increased reduction in leaf elongation in the induced tetraploids during mild drought, it was assumed that the induced tetraploid plants will have a lower wilting score than their diploid progenitors, however, this was true in just one induced tetraploid (Shoot-4x), which had a lower wilting score than the diploid progenitors and a higher reduction in leaf growth. Three induced tetraploids (Grazer-4x, Top speed-4x and Druva-4x) had a higher wilting score despite their high reduction of leaf growth when compared with their diploid progenitors. There was no significant difference between Magloire and Magloire-4x in the leaf growth reduction, but the induced tetraploid had a higher relative water content, Fv/Fm and a lower wilting score. This suggests that the Magloire-4x was able to tolerate drought better than its diploid progenitor. This superior trait demonstrated by Magloire-4x was also apparent in the field experiment and could be associated with many factors, including dosage effect, the neo-functionalisation of duplicated genes, increased allelic diversity and mutation buffering (Comai, 2005; te Beest et al., 2012).

The results from severe drought indicated that the tetraploid cultivars and induced tetraploid lines were able to recover from the stress compared to their diploid progenitors. The diploid cultivars, induced tetraploid lines and tetraploid cultivars suffered extensive wilting after severe drought. The damage caused by drought depends largely on its intensity and the duration. The induced tetraploid lines and tetraploid cultivars recovered better from severe drought than the diploid cultivars (Figure 8). The induced tetraploid lines and tetraploid cultivars also had better recovery time and plant vigour than their diploid counterparts.

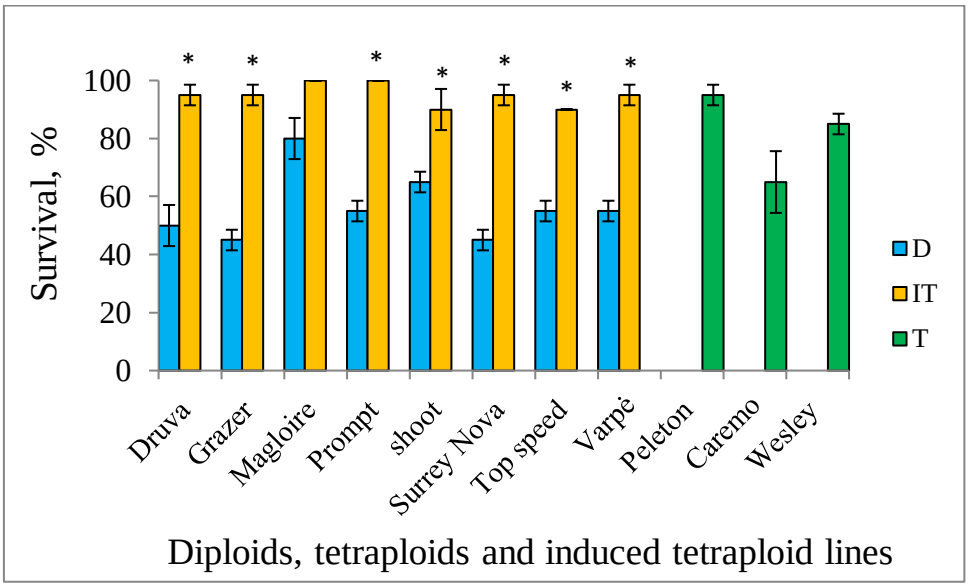


Figure 8. *The survival rate of diploid cultivars (D) induced tetraploid lines (IT) and tetraploid cultivars (T) after five days without watering. Data shown as mean ± standard error of two replicates; the means followed by * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids*

The results from severe drought also indicated a significant positive correlation (Figure 9) between survival rate and antiradical activity at the end of mild drought (r = 0.79, $p \leq 0.05$), and phenolic content (r = 0.72, $p \leq 0.05$). The degree of drought recovery depends on the extent of damage resulting from membrane lipid peroxidation. Lipid peroxidation is considered to be the main cause of membrane oxidative degradation. Cultivars with more antiradical activity are therefore more likely to recover from severe drought (Zhang & Kirkham, 1996).

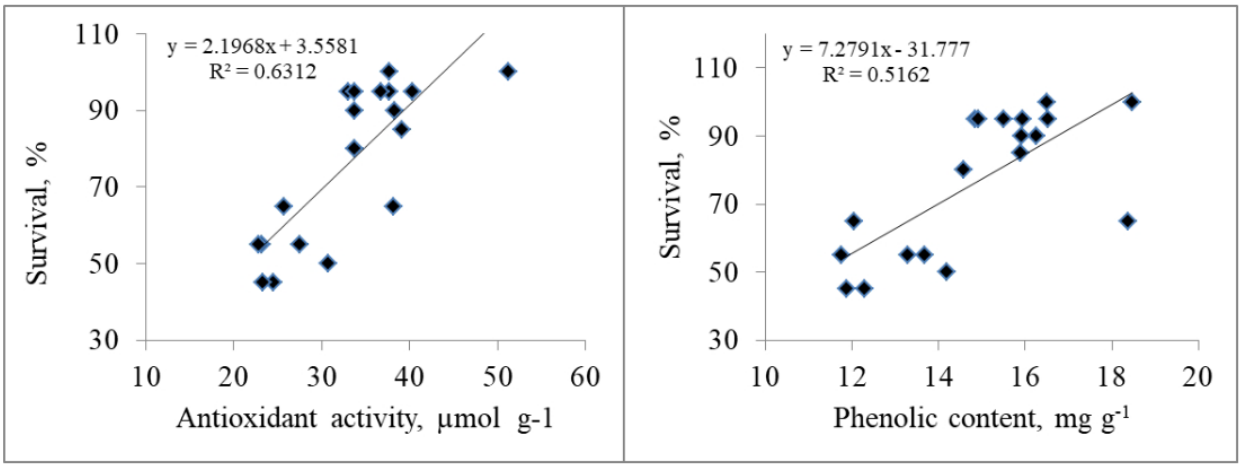


Figure 9. *The relationship between antiradical activity, phenolic contents and survival rate. $p \leq 0.05$*

The summary

ROS generation such as H_2O_2 (hydrogen peroxide), $O_2^{\cdot-}$ (superoxide) and ·OH (hydroxyl) radicals increases when plants are subjected to drought. Excess ROS production can damage cellular structures, proteins, lipids and nucleic acids, and leads to cell death. Antioxidants alleviate the damage caused by ROS. The induced tetraploid lines demonstrated higher antiradical activity and more phenolic contents. There was also a significant positive correlation between antiradical activities, phenolic contents and the survival rate.

Westerwolths ryegrass displayed signs of dehydration avoidance when subjected to a five-day mild drought. Five induced tetraploid lines had a significant reduction in leaf growth compared to their diploid counterparts. Variations were observed in the RWC and chlorophyll fluorescence of both cytotypes, however, an increase in the ploidy level appears to improve the tolerance of Westerwolths ryegrass to severe drought.

3.3. Comparison of the morphological traits between the diploid cultivars, induced tetraploids and tetraploid cultivars in the field trials

Analysis of variance

The year, ploidy level and cultivars were taken as the main factors. The effect of ploidy and cultivars were not significant for the morphological traits but the year effect was highly significant as shown by the F values. This indicated that the different weather condition in 2017 and 2018 had the largest effect on the morphological traits. To determine the effect of ploidy on the morphological traits separately in the years, the induced tetraploid lines and the diploid cultivars were considered as a variable with two levels while fitting replicate as random using a linear mixed model. Result of the interaction between ploidy and cultivar are presented in Table 7.

Table 7. *The effect of ploidy, year and cultivar and their interaction effects on the plant traits between diploids and induced tetraploids as indicated by the F values*

Traits	Year	C (df = 4)	P (df = 1)	R (df = 2)	P × C (df = 4)
Plant height	2017	12.94**	1.30	5.64**	5.64**
	2018	17.96**	12.82**	3.20	40.43**
Flag leaf area	2017	12.62**	1.64	2.26	41.46**
	2018	4.23**	102.66**	0.63	74.15**
Inflorescence length	2017	37.14**	14.74**	0.95	58.04**
	2018	68.72**	168.14**	0.67	177.52**

Y=year, C= cultivar, and P =ploidy level ** indicate significant difference and $p \leq 0.01$

3.3.1. Plant height

No significant difference was noted ($p > 0.05$) in the plant height between the diploids and their corresponding induced tetraploids in the 2017 field trials, except in the cultivar 'Druva', in which the diploids were significantly higher than the induced tetraploid counterpart (Figure 10). The tetraploid cultivars, however, performed better and were significantly higher than both the diploid cultivars and induced tetraploids. In the 2018 field trials, which had lower rainfall and higher average temperatures, significant differences were observed in the plant height of diploid cultivars and the respective induced tetraploid lines ($p \leq 0.05$). Most of the induced tetraploids were taller and showed greater plant vigour than their diploid progenitors.

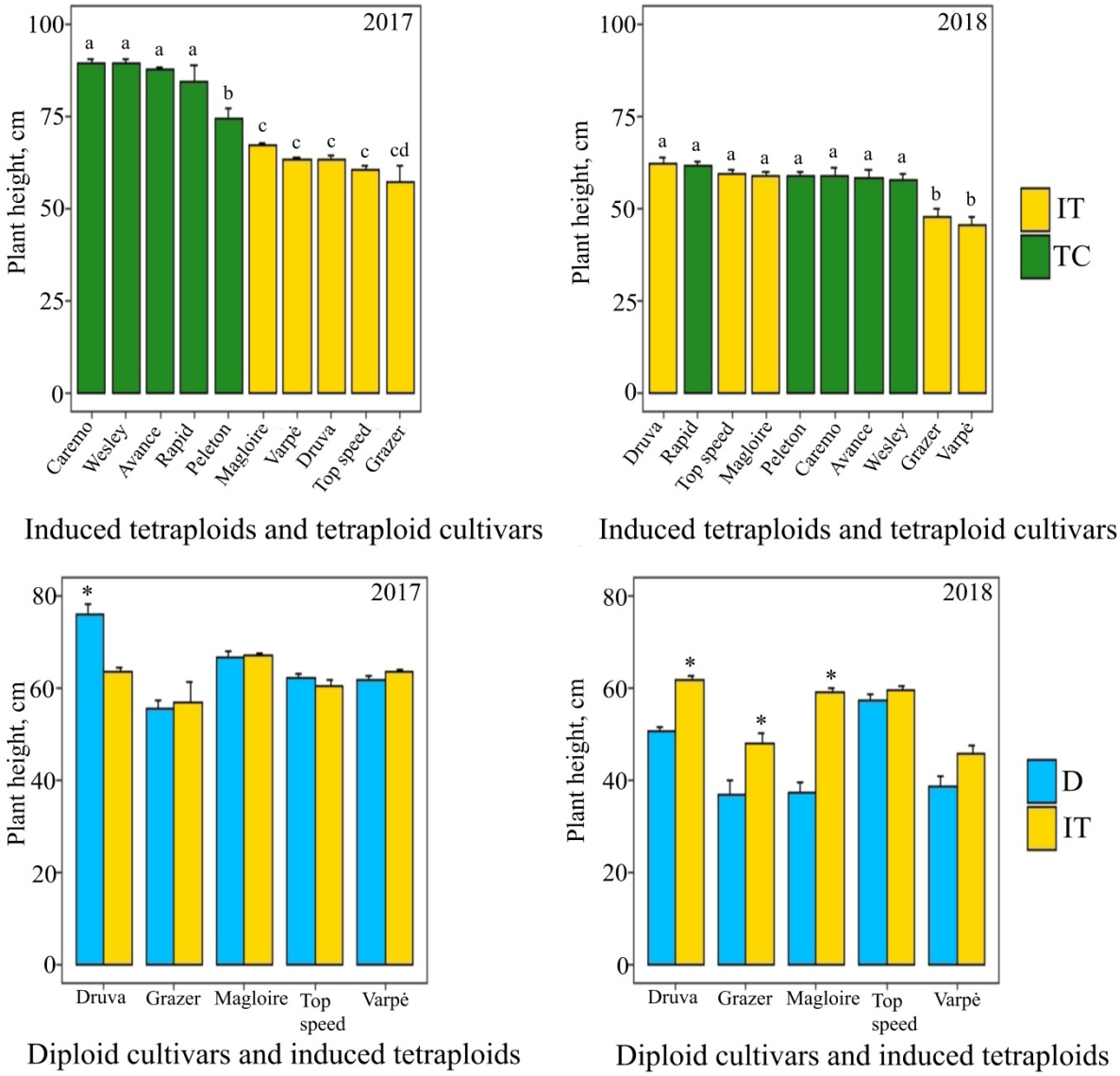


Figure 10. *The plant height of diploid cultivars (D) induced tetraploid lines (IT), and tetraploid cultivars (TC). Data shown as mean ± standard error of 3 replicates. * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids. Different letters indicate significant differences at $p \leq 0.05$. Duncan's multiple range tests.*

The prominence of polyploids in grass species is an indicator that polyploid has some adaptive importance. Polyploidy has been widely studied in many plant species and is known to often demonstrate phenotypes that are not present in the diploid progenitors (Ramsey and Schemske, 2002). These polyploidy induced traits, such as drought tolerance and increased biomass production, could be advantageous in many agricultural processes. Although the mechanism by which the novel traits are manifested in polyploid plants is still not properly understood, neofunctionalisation of genes has been a long standing theory according to which genes acquire a new function after gene duplication (Osborn *et al.*, 2003).

In both years of investigation, the diploid and tetraploid cultivars demonstrated variations in their growth patterns. In 2017 field trials, the tetraploid cultivars were higher than both the induced tetraploid lines and the diploid cultivars. The functional divergence resulting from polyploidisation often confers selective advantages to polyploids (Osborn *et al.*, 2003). These advantages are rarely immediate but occur over a period of time, and this could be a reason why no significant difference was observed in the plant height of induced tetraploid lines and diploid progenitors. Other studies, however, have reported that the effect of polyploidisation is immediate (Renny-Byfield & Wendel, 2014). The tetraploid cultivars may have performed better than the induced tetraploid lines, especially in terms of plant height, as a result of genomic stability and epigenetic changes such as DNA methylation and histone modification, which can be inherited in gene expression (Liu & Wendel, 2003). Dar *et al.* (2013) reported that the DNA methylation changes increased from first generation of induced tetraploids to the fourth generation in *Phlox drummondii*. DNA methylation has also been found to play a role in genome stabilisation and the expression of redundant genes (Lee & Chen, 2001; Adams *et al.*, 2003). DNA methylation also plays an essential role in plant development (Aversano *et al.*, 2013). In essence, the induced tetraploids could perform better in subsequent generations when the DNA methylation increases and the genome stabilises.

3.3.2. Flag leaf area

We evaluated the variations in the area of the flag leaf in diploid cultivars, induced tetraploid lines and tetraploid cultivars, and found induced tetraploid lines and tetraploid cultivars demonstrating a better phenotypic character than the diploid cultivars in both growing seasons. Drought periods in 2018 apparently affected the flag leaf area of both cytotypes, as shown in Figure 11. The flag leaves had smaller surface areas in both cytotypes compared to those in the 2017 field trials. This reduction in the area of the flag leaf is an adaptive response to drought which limits the

assimilating surface in all the diploid cultivars, induced tetraploid lines and tetraploid cultivars. The reduction in the flag leaf area was more profound in the diploid cultivars, however.

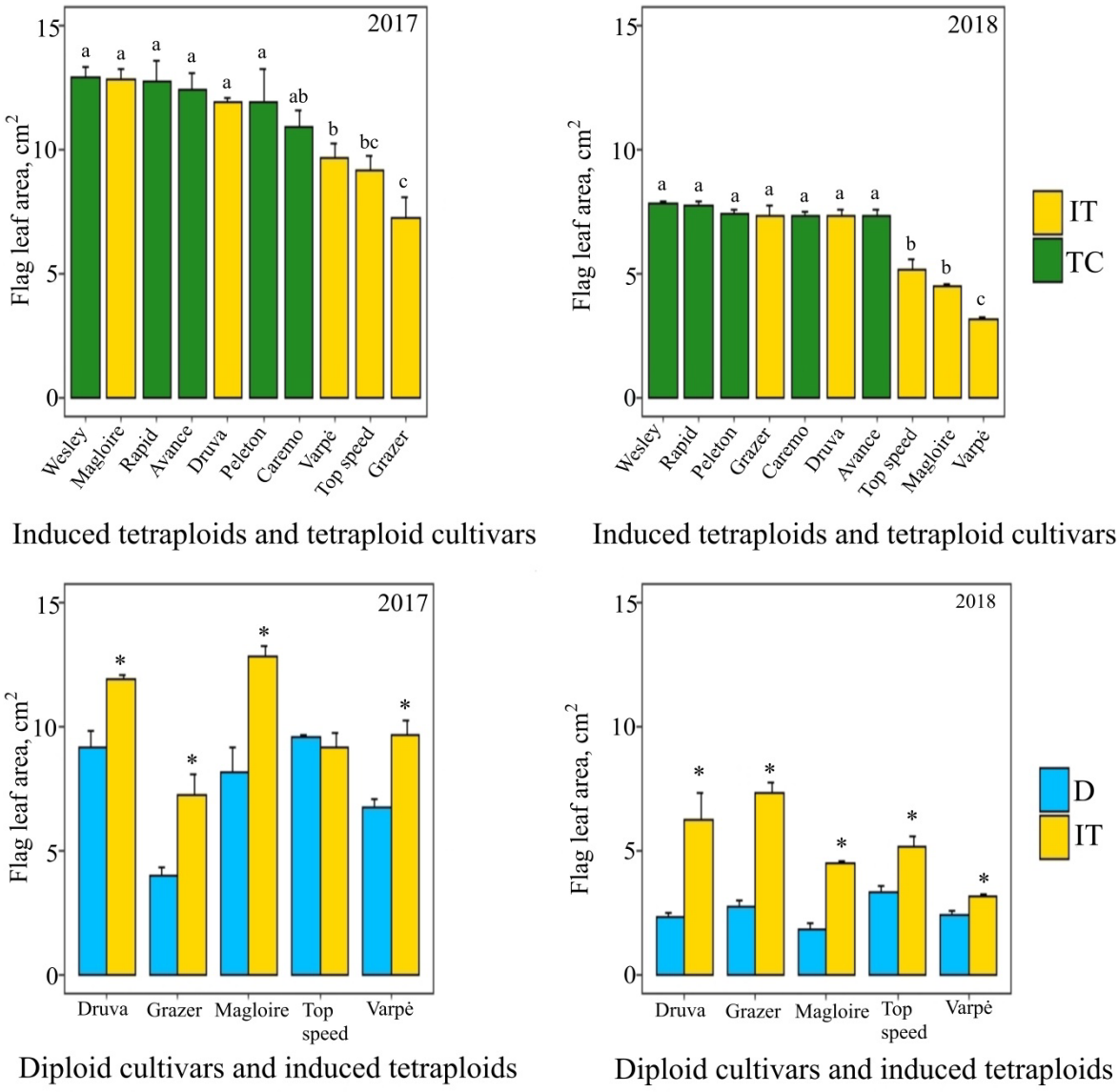


Figure 11. *The flag leaf area of diploid cultivars (D) induced tetraploid lines (IT), and tetraploid cultivars (TC). Data shown as mean ± standard error of 3 replicates. * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids. Different letters indicate significant differences at $p \leq 0.05$. Duncan's multiple range tests*

3.3.3. Flag leaf length

The flag leaf length was significantly affected by drought. In the 2017 growing season, most of the diploid cultivars and induced tetraploids had no significant difference in flag leaf length except in the 'Top speed' and 'Magloire' cultivars, however, most of the induced tetraploids had a longer flag leaf length in the 2018 growing season, as shown in Figure 12.

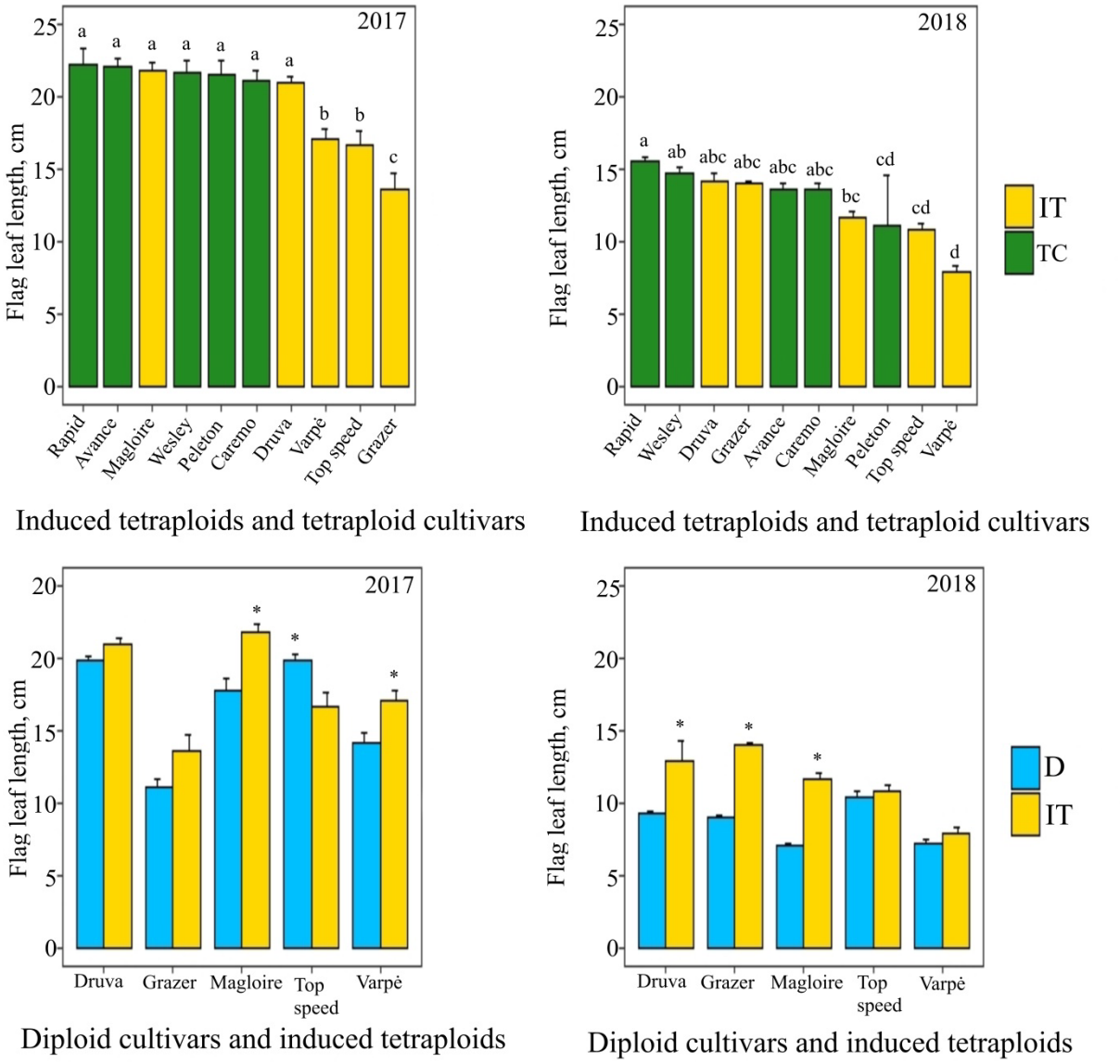


Figure 12. *The flag leaf length of diploid cultivars (D) induced tetraploid lines (IT), and tetraploid cultivars (TC). Data shown as mean ± standard error of 3 replicates. * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids. Different letters indicate significant differences at $p \leq 0.05$. Duncan's multiple range tests*

3.3.4. Inflorescence length

Drought significantly affected the length of inflorescence in the second year of investigation. The induced tetraploids had a longer inflorescence length in both years when compared to their parental diploid, except in the 'Top speed' cultivar, as shown in Figure 13. The difference in the inflorescence length between the induced tetraploid and diploid was even more apparent in the 2018 growing season.

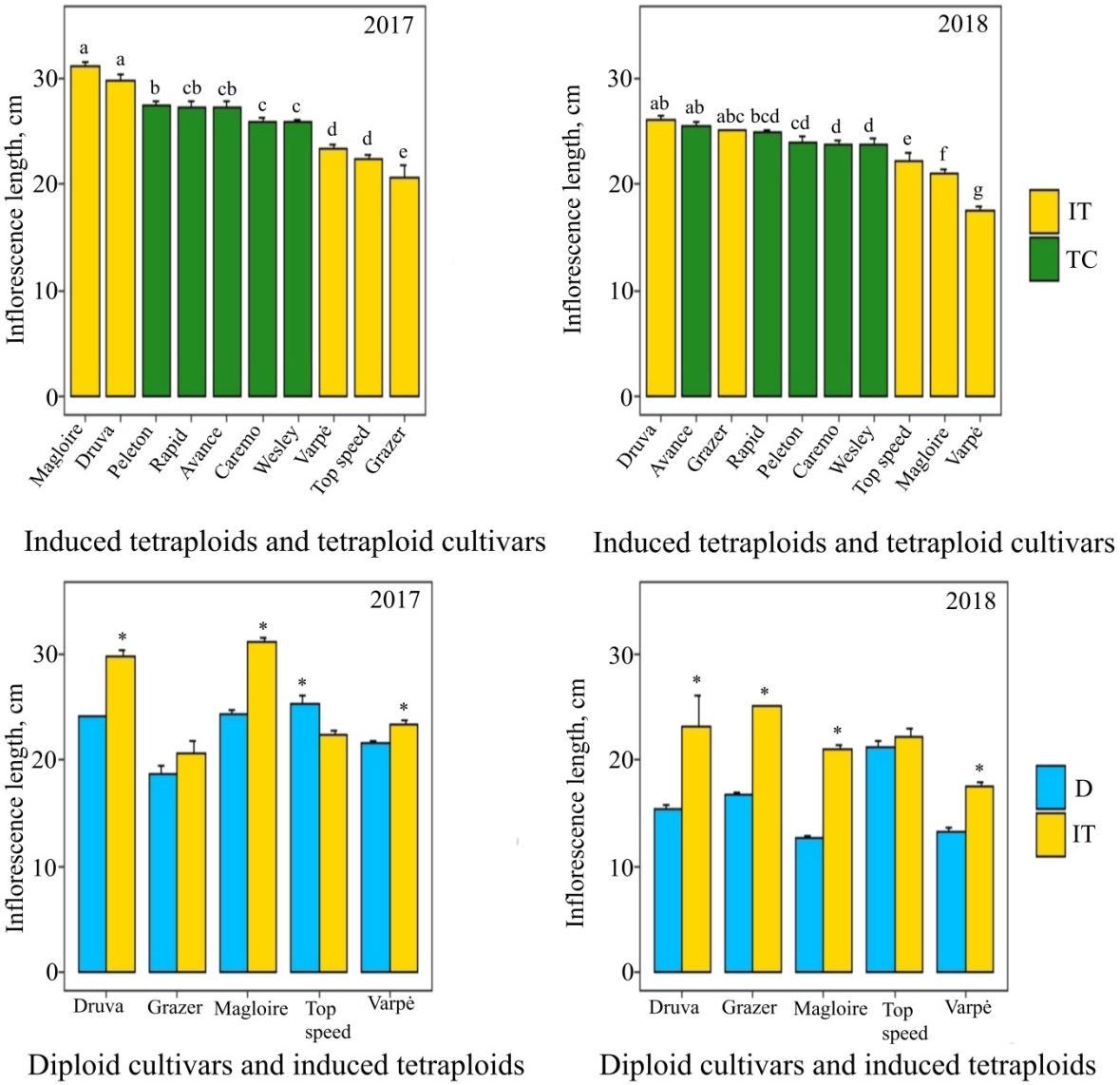


Figure 13. *The inflorescence length of diploid cultivars (D) induced tetraploid lines (IT), and tetraploid cultivars (TC). Data shown as mean ± standard error of 3 replicates. * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids. Different letters indicate significant differences at $p \leq 0.05$. Duncan's multiple range tests*

Leaf growth is a dynamic process often involving independent pathways that direct the cell components (Kalve *et al.*, 2014; Gao *et al.*, 2016). Previous studies have also shown that the autotetraploids of *Lolium* species had long leaves due to the increase in cell length and a faster rate of cell elongation (Sugiyama, 2005). The increase in the flag leaf area of the induced tetraploids compared to the diploid progenitors can probably be attributed to an increase in the size of cells as a result of genome duplication and a faster rate of elongation. It is also possible

that DNA methylation resulting from chromosome duplication could lead to a differential gene expression level resulting in the observed changes in the plant morphology of tetraploids.

We carried out a correlation analysis between the antiradical activity and phenolic contents at the end of mild drought with the morphological traits in 2018 field trials (Table 8) characterized by a lower amount of rainfall. All the morphological traits had a significant medium positive correlation with the antiradical activity and phenolic contents ($p \leq 0.01$).

Table 8. *Correlation coefficients matrix between physiological response during mild drought and morphological traits in the 2018 growing season*

	Plant height	Flag leaf area	Inflorescence length
Antiradical activity	0.49**	0.46**	0.41**
Phenolic content	0.53**	0.53**	0.51**

** Correlation is significant at the $p \leq 0.01$

The phenotypic response of plants to stress are complex and governed by the interactive effects of factors such as stress duration, genotypes, developmental stage at which the stress occurs and the intensity of the stress (Obidiegwu *et al.,* 2015). Drought is a complex quantitative trait and previous studies have shown that drought tolerance could vary at different developmental stages (Kron *et al.,* 2008). Yet, Boutraa *et al.* (2010) reported that genotypes exhibiting high DPPH scavenging activity and high phenolic content at the seed stage continued to show high antiradical activity and phenolic contents at other stages when working on drought tolerance in wheat cultivars. This is a drought tolerance mechanism. In this study, the induced tetraploid lines had more antiradical activity and phenolic contents in response to mild drought. This difference in physiological response at the seedling stages could proceed to other developmental stages and contribute to the observed improved performance of the induced tetraploid lines in the 2018 field trials. However, future work will be done on the DPPH scavenging and total phenolic contents in field trials at various developmental stages.

When comparing results from cultivars in field experiments (2018) and the drought simulation experiment, it is not surprising that the induced tetraploids with a lower wilting score after the mild drought experiment showed a better phenotypic character in the field experiments, with the exception of Grazer-4x and Druva-4x which had high wilting scores and a better phenotypic character than their diploid progenitors.

Homeostasis can be described as the ability of organisms to adjust their internal physiological condition when responding to a changing external environment (Wang *et al.,* 2016). In this regard, our empirical data on the phenotypic traits suggested that the tetraploid plants could be at a

homeostatic advantage over their diploid counterparts, indicating a physiological response that confers advantages to tetraploids during drought conditions.

The summary

Diploid, tetraploid cultivars and the induced tetraploid lines of Westerwolths ryegrass were established in field trials during the 2017 and 2018 growing seasons. The plant height, flag leaf length, flag leaf area and inflorescence length of both cytotypes were compared. No significant difference was found in the plant height of the diploid cultivars and their respective induced tetraploid lines in the 2017 growing season, however the tetraploid cultivars were taller than the induced tetraploid lines. Epigenetic changes and genomic instability resulting from polyploidisation were thought to contribute to the performance of the induced tetraploid lines in 2017. The plant height, flag leaf length, area, and inflorescence length of the induced tetraploid lines demonstrated superior phenotypic features in 2018, which was characterised by increased average temperature and reduced rainfall, suggesting the increase in ploidy level contributed to the superior performance in most of the induced tetraploid lines.

3.4. Dry matter yield (DMY) of diploid cultivars and induced tetraploid lines

Generally, there is an increase in the demand for forage to meet animal nutrient requirements. Many approaches have been taken over the years to increase annual ryegrass production. One is crop management. Crop management involves techniques such as selecting high yielding disease-resistant cultivars, applying the optimum amount of fertiliser and irrigating adequately. This approach is simple and quick, however, breeding programs are innovation-based, focused on developing plants that are better adapted and have economic benefits. In polyploid plants, an increase in chromosome number, genomic interaction, and genetic alteration usually leads to plants with superior characteristics. This makes polyploidisation a credible approach to crop improvement. Polyploid inductions have been undertaken in many plants and have been found to improve plant productivity.

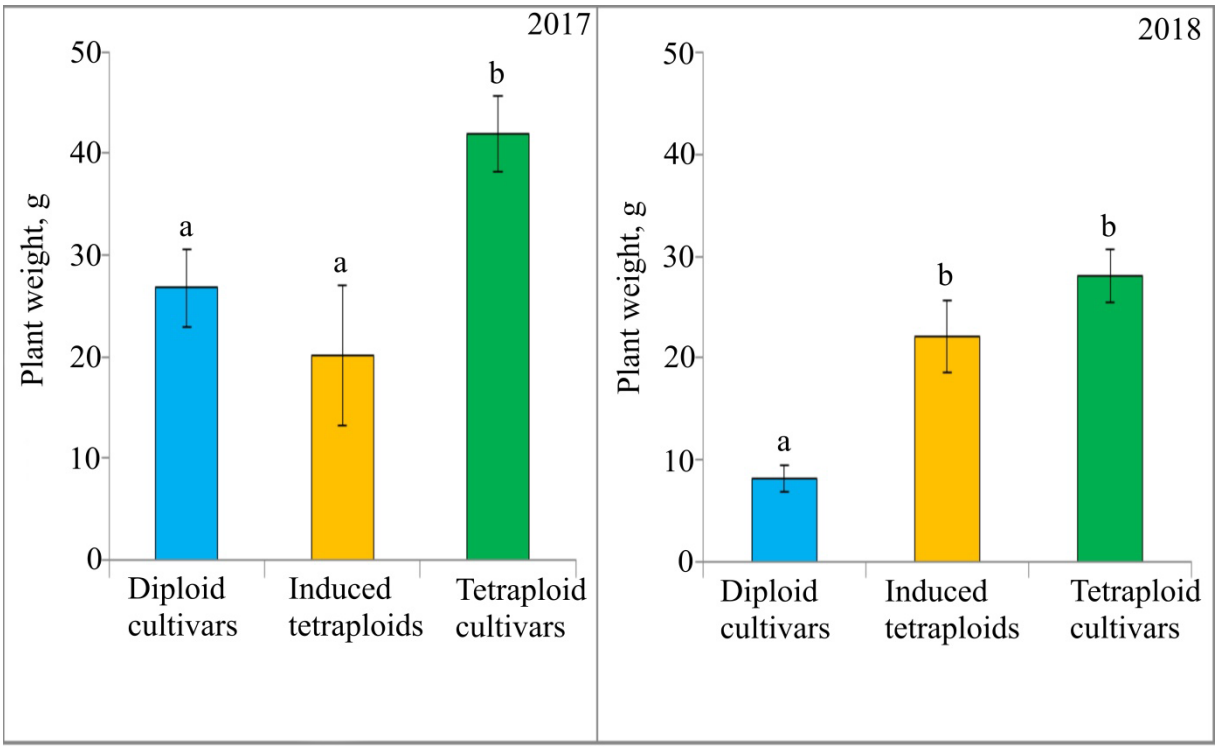


Figure 14. *The dry matter yield in 2017 and 2018 of diploid cultivars, induced tetraploid lines and tetraploid cultivars. Data shown as mean ± standard error. Different letters indicate significant differences at $p \leq 0.05$. Duncan's multiple range tests*

As seen in Figure 14, the induced tetraploid lines did not produce higher dry matter yield than the diploid cultivars in 2017, however, the tetraploid cultivars produced more dry matter yield than both the diploid cultivars and induced tetraploids. Although drought appeared to reduce the DMY of the diploid cultivars, and tetraploid cultivars in 2018, the induced tetraploid lines appeared to be more stable and performed better than their diploid progenitors, producing significant higher dry matter yields.

Many genetic and epigenetic changes take place in natural and induced chromosome duplication. Genetic changes include point mutations, gene conversion, aneuploidy, structural chromosome rearrangements and loss of duplicated genes. On the other hand, the epigenetic changes could be as a result of the modification in the chromatin compaction levels, such as histone acetylation, DNA methylation, RNA interference and dosage compensation (Osborn *et al.*, 2003). Epigenetic changes are capable of altering the gene expression levels without changing the DNA sequence. For example, Lee & Chen (2001) suggested that the epigenetic changes in the synthetic polyploids of *Arabidopsis* Heynh affected the gene expression pattern, causing new phenotypes to emerge.

Renny-Byfield and Wendel (2014) suggested that immediate and long-term disturbances in the genome, transcriptome, and epigenome are intrinsic to polyploidy. This may explain why the induced tetraploid lines did not produce higher DMY than their respective diploid progenitors in

the 2017 growing season. The genomes of polyploid plants stabilise as the generation progresses, and high levels of heterozygosity are usually expected in autotetraploids compared to their diploid counterparts, due to polysomic inheritance (Soltis *et al.,* 2003). This high level of heterozygosity has been positively correlated to increments in vigour in alfalfa (Katepa-Mupondwa *et al.,* 2002). The increase in ploidy level appears to increase the tolerance of Westerwolths ryegrass to abiotic stress. The induced tetraploid lines produced more DMY than the diploid progenitors in the 2018 growing season, characterised by higher average temperatures and extended period of drought.

3.5. Crown rust infection

Crown rust is a fungus disease caused by *Puccinia coronata*. This disease has been widespread for many years in the western and northern parts of Europe (Jonsson *et al.,* 1998) mostly observed from late summer to autumn and prevalent when grasses are depleted of nutrients. Crown rust is the most damaging foliar disease of ryegrasses and results in the significant loss in dry matter and quality from pastures used in milk and meat production. This disease not only affects ryegrass yield but also affects the composition and sward quality (Arojju *et al.,* 2018). A ten-year study (2008–2018) of crown rust infection on Perennial ryegrass and Festulolium in Lithuania showed that the infection occurred mostly in early autumn (September). No infection was observed in 2011 and 2015, but in 2012, 2017 and 2018 the infection appeared unexpectedly early, indicating that changing climate increases the risk of infection in the future (Kemešytė *et al.,* 2019).

The onset of rust diseases results from a failure of the host plant's immune system to recognise the pathogen and activate defence responses (Dangl, 1995). This disease can be controlled either with the use of fungicides or breeding resistant genotypes. The resistant genotypes have receptor proteins that are predominantly encoded by nucleotide binding site-leucine rich repeat (NBS-LRR) genes (Dangl *et al.,* 1996). They also recognise elicitor molecules specific to the pathogen strains (Dangl & Holub, 1997).

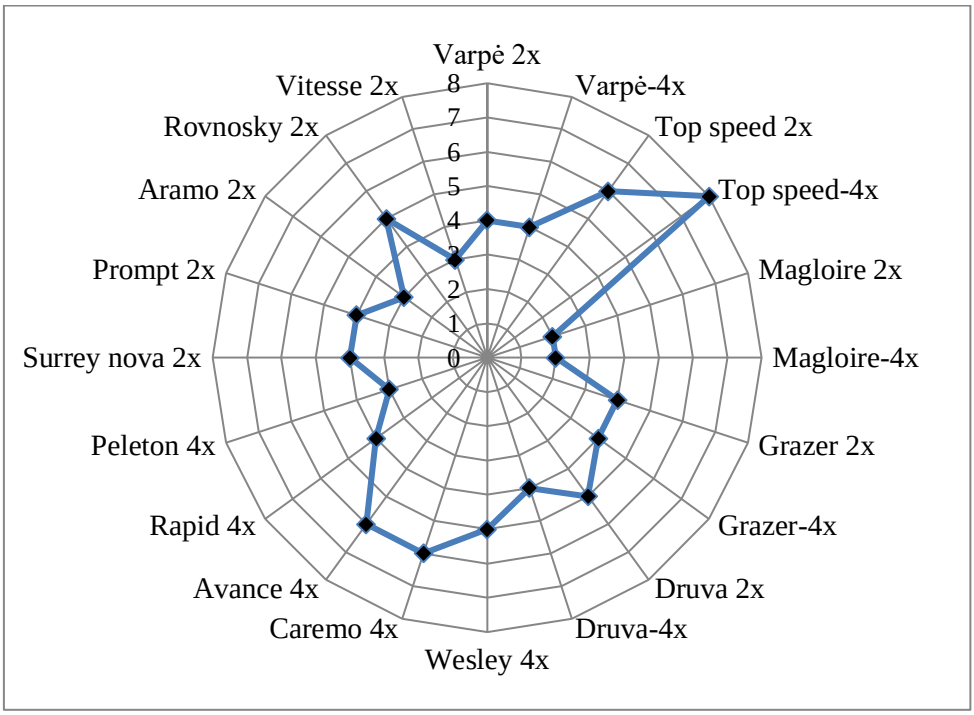


Figure 15. *Visual scoring for crown rust infection on Westerwolths ryegrass cultivars and induced tetraploid lines in the 2018 growing season*

Note: 1 = no rust, 2 = trace of rust, 3 = 5% of the foliage covered with rust, 4 = 10% of the foliage covered with rust, 5 = 25% of the foliage covered with rust; predominantly leaves with scattered pustules, 6 = 40% of the foliage covered with rust leaves + spotted with many pustules, 7 = 60% of the foliage covered with rust; leaves densely covered with areas of rust and few necrosis, 8 = 75% of the foliage covered with rust; leaves densely covered with rust and many necrotic leaves, 9 = more than 75% of the foliage covered with rust; predominantly leaves with necrosis.

Crown rust significantly affected the plants in the field, especially during the 2018 growing season. The infection was observed in the cultivars in varying degrees irrespective of their ploidy. 'Magloire' (4x and 2x) were the most resistant to crown rust and induced tetraploid 'Top speed' (4x) was severely affected by crown rust as shown in Figure 15. Even within cultivars, some genotypes were found to be more resistant to the disease than the others, suggesting that resistance to crown rust depends more on genotype than on the ploidy level.

Summary

The high dry matter yield and quality associated with resistance to drought and rust is one of the most important criteria in Westerwolths ryegrass breeding. Yield trials in 2017 and 2018 clearly demonstrated an inhibition of dry matter production in the diploid cultivars and the respective induced tetraploid lines in response to drought. The induced tetraploid lines produced more DMY than their diploid progenitors, suggesting that the increase in ploidy level affected tolerance to drought.

Fungicides are able to reduce the damage caused by Puccinia coronata, however this comes at environmental and economic cost, and risks to human health. Developing resistant cultivars is therefore desirable. Cultivar 'Magloire' (4x and 2x) is the most resistant to crown rust, and could therefore be used in breeding programs for crossing and development of new resistant populations, cultivars.

3.6. Genome duplication effect on the germination of Westerwolths ryegrass seeds under salt stress

Seeds from both cytotypes varied in length and weight. The induced tetraploid seeds were longer and heavier than their diploid progenitors (Table 9).

Table 9. *The seed length and weight of diploid cultivars and respective induced tetraploid lines of Westerwolths ryegrass*

Diploid cultivar / induced tetraploid line	Seed length mm	1000 seed weight g
Magloire	5.83 ± 0.17	2.77 ± 0.07
Magloire-4x	7.17 ± 0.17*	4.03 ± 0.05*
Druva	6.00 ± 0.00	2.39 ± 0.06
Druva-4x	7.00 ± 0.29*	4.23 ± 0.11*
Varpė	5.50 ± 0.00	2.81 ± 0.10
Varpė-4x	8.33 ± 0.61*	4.95 ± 0.06*
Grazer	5.00 ± 0.29	2.81 ± 0.08
Grazer-4x	6.50 ± 0.51*	4.60 ± 0.08*
Prompt	5.17 ± 0.17	2.70 ± 0.09
Prompt-4x	6.17 ± 0.17*	3.78 ± 0.05*
Shoot	5.67 ± 0.17	2.19 ± 0.08
Shoot-4x	7.33 ± 0.34*	4.08 ± 0.11*
Surrey nova	4.83 ± 0.17	2.93 ± 0.10
Surrey nova-4x	6.67 ± 0.17*	3.89 ± 0.08*

Note. Data shown as mean ± standard error of three replicates; the means followed by * between diploid cultivar and corresponding induced tetraploid line are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

The effect of salinity stress on germination is shown in Figure 16 and Table 10. Salinity stress appears to delay the outset of germination or inhibit germination in both cytotypes and across the cultivars. The inhibition of germination also increased as the salinity concentration increased ($r = 0.86$, $p \leq 0.01$).

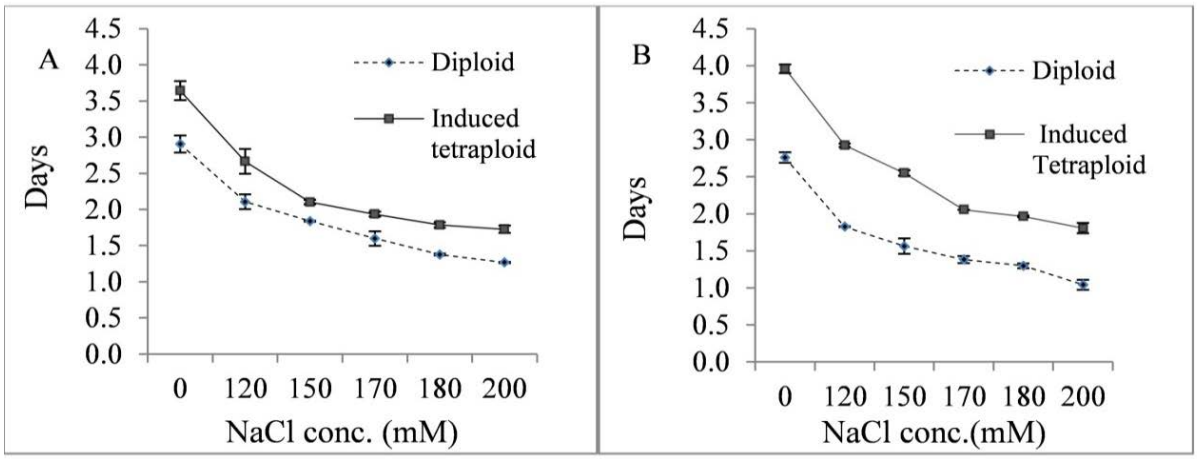


Figure 16. *Germination index of diploid cultivars and respective induced tetraploid lines 'Magloire' (A) and 'Varpė' (B) of Westerwolths ryegrass in different NaCl concentrations. The error bars represent the standard error of the mean*

The results showed that germination parameters were reduced overall in the seeds of both diploid cultivars and the induced tetraploids compared with their respective control, however, chromosome duplication appears to have a role in improving the tolerance to salinity stress at the germination stage, as seen in the germination index and T50 values. The germination percentage, mean germination time, T50, germination index and germination value were calculated for both cytotypes. Cultivar differences also contributed to the observed differential response to the salinity treatments.

The germination process begins with seed imbibition, in which water is absorbed by seeds to make the nutrients in the endosperm available. Imbibition is a critical stage in the germination process and has to be completed before germination occurs. The imbibition process depends on several factors, including the temperature, water, oxygen, permeability of the seed coat, seed size and osmotic potential (Louf *et al.,* 2018). Several studies have shown that germination and seedling growth are significantly affected by salt stress; however, little is known about the role that ploidy has in Westerwolths ryegrass (Matthews & Khajeh-Hosseini, 2007; Ahmed *et al.*, 2017). Studies have also reported that imbibition is completed faster in the smaller seeds within the same ploidy level because of the increase in surface area to volume ratio, hence increasing the absorption of water and leading to faster germination (Schneider, 1998; Souza & Fagundes, 2014).

Other studies, however, have reported that tetraploid seed germinated faster than its diploid counterparts (Eliášová & Münzbergová, 2014).

Table 10. *Germination percentage, mean germination time, germination index and T50 of different diploid cultivars and the respective induced tetraploids of Westerwolths ryegrass after salinity (200 mM NaCl) treatment*

Diploid cultivar / induced tetraploid line	Germination %	Mean germination time, days	Germination index, days	T50, days
Magloire (control)	91.67 ± 2.33 abc	4.50 ± 0.00 fg	2.93 ± 0.22 cd	3.93 ± 0.07 fghi
Magloire-4x (control)	93.33 ± 0.00 abc	3.93 ± 0.07 h	3.62 ± 0.09 ab	3.45 ± 0.05 i
Magloire	63.33 ± 3.33 de	8.00 ± 0.09 bc	1.29 ± 0.04 gh	7.50 ± 0.00 ab
Magloire-4x	90.64 ± 3.33 abc	7.48 ± 0.05 c	1.87 ± 0.09 f	6.58 ± 0.25 c
Druva (control)	91.67 ± 2.33 abc	4.33 ± 0.00 gh	3.18 ± 0.38 bc	3.74 ± 0.01 ghi
Druva-4x (control)	98.00 ± 3.33 ab	4.38 ± 0.02 gh	3.37 ± 0.11 bc	3.85 ± 0.08 fghi
Druva	46.67 ± 0.00 ef	6.64 ± 0.07 d	1.10 ± 0.00 hi	7.58 ± 0.04 ab
Druva-4x	53.33 ± 0.00 ef	6.88 ± 0.13 d	1.18 ± 0.01 h	6.33 ± 0.04 cd
Varpė (control)	80.00 ± 0.00 bcd	4.75 ± 0.09 fg	2.56 ± 0.04 de	4.31 ± 0.06 efg
Varpė-4x (control)	100.00 ± 0.00 a	3.90 ± 0.04 h	3.88 ± 0.04 a	3.44 ± 0.02 i
Varpė	46.67 ± 6.66 ef	8.23 ± 0.11 ab	0.88 ± 0.14 hi	7.88 ± 0.38 ab
Varpė-4x	84.25 ± 3.47 abc	6.83 ± 0.08 d	1.71 ± 0.09 fg	6.25 ± 0.05 cd
Grazer (control)	82.30 ± 5.87 abc	5.35 ± 0.44 e	2.40 ± 0.10 e	4.58 ± 0.20 e
Grazer-4x (control)	95.67 ± 3.33 ab	4.93 ± 0.14 ef	3.01 ± 0.03 cd	4.43 ± 0.15 ef
Grazer	38.42 ± 2.34 f	7.88 ± 0.28 bc	0.70 ± 0.04 i	7.52 ± 0.15 ab
Grazer-4x	65.33 ± 2.34 de	6.73 ± 0.21 d	1.21 ± 0.03 h	6.24 ± 0.08 cd
Prompt(control)	98.00 ± 3.33 ab	4.25 ± 0.11 gh	3.53 ± 0.27 ab	3.64 ± 0.06 hi
Prompt-4x (control)	100.00 ± 0.00 a	4.50 ± 0.10f g	3.40 ± 0.06 bc	3.84 ± 0.09 fghi
Prompt	78.37 ± 5.87 cd	6.58 ± 0.18 d	1.81 ± 0.19 f	6.08 ± 0.42 cd
Prompt-4x	56.45 ± 5.93 e	8.04 ± 0.24 b	1.09 ± 0.23 hi	7.42 ± 0.09 ab

Note. Data shown as mean ± standard error of three replicates; the means followed by the same letter within each column are not significantly different ($p > 0.05$, Duncan's multiple range test).

The induced tetraploid seeds of Westerwolths ryegrass were bigger and heavier than their parental diploids, and in most cases germinated faster than the diploid counterparts. This raises the

question of whether an increase in the ploidy level confers an advantage in seed germination and even more, under stress conditions.

The performance of seeds can be seen by comparing the germination parameters of diploids and induced tetraploids. The mean germination time (MGT) for both cytotypes varied across the cultivars and induced lines, and clearly did not explain the role of ploidy in seed germination. Although some researchers have used the MGT to evaluate the seed vigour of many plants (Mavi *et al.,* 2010; Matthews *et al.,* 2012; Chen *et al.,* 2013), other studies found the MGT to be inaccurate, arguing that seeds can have different final germination percentages, and the same MGT, because seeds can germinate across a different spread (Kader, 2005). The MGT better reflected the day on which most of the seeds germinated in a seed lot, and accurately defined as the mean lag period between the start of imbibition and germination for each seed (Matthews & Khajeh-Hosseini, 2007). On the other hand, the median germination time (T50) gave a better understanding of the speed of germination (Soltani *et al.*, 2015). Our results indicated that the induced tetraploids had lower T50 values, especially during the salinity stress, except in the cultivar 'Prompt'.

The germination index gives a more accurate measurement of the germination than germination percentage and the mean germination time, because it takes cognisance of the germination percentage, speed of germination and the spread of germination (Javaid *et al.,* 2018). Our results clearly demonstrate that the induced tetraploid seeds had a higher germination index than their diploid progenitors, both in the control and under stress conditions, except for the cultivars 'Prompt' and 'Druva'. The seeds of diploid cultivar 'Prompt' only had a higher germination index than the induced tetraploid counterpart during salinity stress, while no significant difference was found in the germination index of diploid and induced tetraploid seeds of 'Druva' in either the control or under salinity stress.

Generally, the effect of salinity on seed germination occurs via the ionic toxicity, osmotic effect or a combination of both effects (Zhou & Xiao, 2010; Panuccio *et al.,* 2014). Salinity often increases the osmotic potential while decreasing the water potential, making water unavailable to plants. The lower the osmotic potential in seeds, the more the seed can absorb water and complete imbibition. Zhang *et al.* (2010) further explained that seeds in saline conditions can have decreased osmotic potential when salt is excluded from the cells while using other organic solutes as osmolites to maintain the osmotic potential. Bigger seeds at a higher ploidy level are at an advantage here, compared to smaller seeds at a lower ploidy level, as they have more carbon reserves and can generate a lower osmotic potential and thus, alleviate the need to absorb sodium.

Alternatively, seeds can accumulate and use sodium and chloride ions as osmolites, while having a mechanism that neutralises their toxic effect. The mechanism used by Westerwolths ryegrass to maintain a water potential gradient during the salinity remains unclear.

Our results showed a medium positive correlation (r = 0.55) between antiradical activity during salinity stress at the seedling stage and in the germination index. Interestingly, the increase in antiradical activity during salinity stress at the seedling stage in the induced tetraploid lines was significant when compared to their parental diploid, except in the cultivar 'Prompt', which also had a higher germination index compared to its corresponding induced tetraploid. While some studies have used salinity tolerance at the germination and seedling stages as an indicator for screening tolerant genotypes (Carpici *et al.,* 2010; Shahid *et al.,* 2012; Ravelombola *et al.,* 2017), other studies indicated the opposite, suggesting that the tolerance to salinity stress might be specific to various developmental stages (Lauchli & Epstein, 1990). The relationship between the antiradical activity at the seedling stage and the germination index alone cannot fully explain whether the tolerance at the germination stage in Westerwolths ryegrass could be an indicator of tolerance at other developmental stages. More studies involving physiological responses at various developmental stages are needed to understand how seeds reduce their osmotic potential in the germination process during salinity stress, and also to determine an effective tolerance screening stage in Westerwolths ryegrass.

3.7. Genome duplication effect on the seedling growth of Westerwolths ryegrass under high saline conditions

The seedlings of both diploid and tetraploid Westerwolths ryegrass (three weeks old) were grown in controlled conditions. New unfolding leaves were measured in this experiment. The leaf elongation was determined during stress treatment and in the control in non-destructive daily leaf length measurements. The leaf elongation was measured at the onset of the stress until the end of the experiment. The effect of high salinity treatment was apparent in both induced tetraploids and the diploid cultivars, with a significant reduction in the leaf growth and shoot development. Our results showed that the induced tetraploids had longer leaves compared to their parental diploids during the salinity treatment (Figure 17A). The induced tetraploids also showed a higher reduction in their leaf elongation than their diploid progenitors compared with their respective control experiment (Figure 17B). Differences in the cultivar leaf length and leaf growth reduction were also observed.

In the vegetative stage, the first effect of salinity stress occurs in the root systems of plants and this impairs growth due to the osmotic stress. Osmotic stress reduces the availability of water to

plants and also generates ROS (Ashraf & Foolad, 2013). One of the first metabolic responses of plants under stress is the growth inhibition and downregulation of energy metabolism, suggesting that plants conserve energy (Cramer *et al.*, 2011). The reduction in growth, especially in the leaf area, usually occurs by inhibiting protein synthesis. This is an avoidance mechanism that helps to reduce water loss via transpiration (Rodriguez *et al.,* 2005). Our results show that while the induced tetraploids have longer leaves than their diploid counterparts, the induced tetraploids were able to slow leaf growth more than their diploid progenitors.

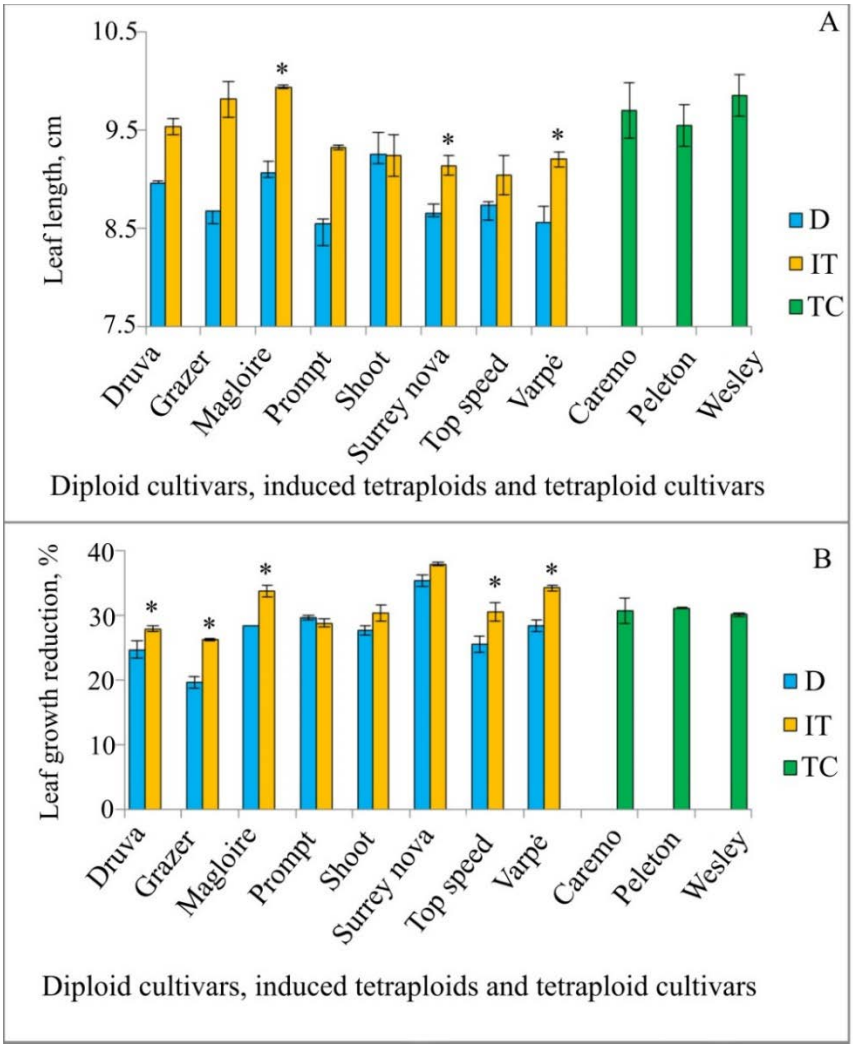


Figure 17. *Leaf length (A) and reduction in leaf elongation (B) in the seedlings of Westerwolths ryegrass treated with 500 mM NaCl. D, IT, TC represents the diploid cultivars, induced tetraploids and tetraploid cultivars, respectively. Data shown as mean ± standard error of three replicates; the means followed by * Represents significant difference at $p \leq 0.05$ pairwise t-test between diploids and corresponding induced tetraploids*

Plants subjected to salinity stress showed signs of wilting compared to the control. Salinity stress also reduced the relative water content. The relative water content varied across the cultivars and most of the induced tetraploid lines were found to have higher relative water content compared to

their diploid progenitors during salinity stress (Table 11). The differences observed between the means were significant.

3.8. Genome duplication effect on the antiradical activity of Westerwolths ryegrass under salt stress

The antiradical activities in response to the salinity stress were determined in both diploids and tetraploids. The results showed that the induced tetraploids produced a greater antiradical activity response than their diploid progenitors, except in the cultivar 'Prompt'. A significantly positive correlation was also found between the antiradical activity and the germination index ($r = 0.55$, $p \leq 0.05$), and the reduction in leaf elongation during salinity stress ($r = 0.64$, $p \leq 0.05$).

Table 11. *The effect of salinity stress on the relative water content (RWC) and the antiradical activity response in diploid cultivars and the respective induced tetraploid lines of Westerwolths ryegrass seedlings*

Diploid cultivar / induced tetraploid line	RWC %	Antiradical activity (control) µmol TE g^{-1}	Antiradical activity (500 mM NaCl) µmol TE g^{-1}
Varpė	68.8 ± 0.19 g	23.7 ± 3.22 de	35.0 ± 0.78 fg
Varpė-4x	75.9 ± 0.49 de	35.4 ± 1.34 a	47.3 ± 0.95 ab
Druva	80.2 ± 1.18 c	32.7 ± 1.01 ab	36.6 ± 1.35 g
Druva-4x	71.8 ± 0.5 f	29.6 ± 1.35 bc	44.3 ± 0.98 bc
Magloire	69.1 ± 0.31 g	33.4 ± 1.81 ab	41.5 ± 2.63 cd
Magloire-4x	80.6 ± 1.01 c	33.2 ± 1.15 ab	49.2 ± 0.11 a
Grazer	75.9 ± 1.43 de	23.0 ± 0.15 e	28.8 ± 2.28 h
Grazer-4x	74.4 ± 0.93 ef	24.7 ± 0.55 de	39.0 ± 1.97 de
Surrey Nova	87.6 ± 1.02 a	27.3 ± 0.54 cde	32.8 ± 1.34 fg
Surrey Nova-4x	83.5 ± 1.01 b	23.3 ± 2.87 de	42.7 ± 2.33 c
Prompt	73.7 ± 0.21 ef	27.8 ± 0.72 cd	36.5 ± 1.77 ef
Prompt-4x	81.0 ± 1.01 bc	28.1 ± 0.02 cd	38.8 ± 2.35 de
Top speed	77.4 ± 0.69 d	27.5 ± 0.50 cde	35.3 ± 1.45 efg
Top speed-4x	88.1 ± 0.63 a	24.0 ± 0.78 de	41.3 ± 1.23 cd
Shoot	74.0 ± 1.00 ef	30.0 ± 0.52 bc	35.7 ± 0.55 efg
Shoot-4x	80.4 ± 0.36 c	32.8 ± 1.14 ab	43.2 ± 0.89 c

Note. Data shown as mean ± standard error of three replicates; the means followed by the same letter within each column are not significantly different ($p > 0.05$, Duncan's multiple range test) TE – Trolox equivalent.

Prolonged exposure to salinity stress leads to ion toxicity and nutrient imbalance. This often results in sodium toxicity and the generation of reactive oxygen species (ROS) (de la Torre-González *et al.,* 2017). Plants growing in an optimal condition are said to be redox homeostatic, because there is equilibrium in the production and scavenging of ROS. When plants generate high levels of ROS with an inefficient mechanism for scavenging the ROS, it causes an imbalance in the cellular redox, which leads to oxidative stress (Sharma *et al.,* 2012). Plants generally combat high levels of ROS by activating the enzymatic and non-enzymatic system that scavenges the ROS. The enzymatic system involves enzymes such as superoxide dismutase, peroxidase, catalase, glutathione reductase and ascorbate peroxidase (Huseynova *et al.,* 2014; Caverzan *et al.,* 2016). The non-enzymatic system involves tocopherols, glutathione and ascorbic acid, which are also involved in ROS detoxification (Caverzan *et al.,* 2016).

The synthesis of the antioxidants and their activities are altered when plants are subjected to stress conditions. Polyploids can increase their tolerance to stress by altering their physiology, phenology and morphology (Adams & Wendel, 2005). Reports have shown that the increase in stress tolerance correlates with the increase in antiradical activity (Aghaei *et al.,* 2009). Our results indicated that the induced tetraploid lines responded with significantly higher antiradical activity than their diploid progenitors, except in the cultivar 'Prompt'. This is similar to the report by Meng *et al.* (2011), where increased antiradical activity contributed to the tolerance of auto-induced tetraploid turnips over their diploid progenitors. Our results also showed a positive correlation between reduction in growth and the antiradical activity, suggesting that the tetraploid lines are better adapted in their first response to salinity stress.

Induced tetraploid lines in most cases had a higher RWC compared to their corresponding diploid progenitors. The response to salinity stress involves a cascade of reactions involving many genes at the molecular level, however, the duplication of the genetic material seems to have an advantage over the diploid in the first response to salinity in Westerwolths ryegrass.

The summary

Soil salinisation remains of huge concern in the realisation of sustainable agricultural production. While the emphasis has been placed on food crops, forage production, which is an important component of the food chain, is also affected.

The effect of different salt concentrations at the germination and seedling stages was studied. The results showed that seeds from the induced tetraploid lines, despite being bigger, had higher germination index and lower T50 values compared to the diploid progenitors. At the seedling

stage, an increase in the ploidy level played a role in conferring improved tolerance to salinity stress. The induced tetraploid lines were at an advantage over their diploid counterpart as the tetraploid plants significantly reduced their growth, had a higher relative water content, and had more antiradical activities in response to salinity stress.

3.9. Differential expression of drought related genes in two diploid cultivars and their respective induced tetraploid line

The molecular response to drought, especially the differential expression of genes at different time-points of water deficit, has been studied in *Arabidopsis thaliana* (Seki *et al.,* 2002) and in other plant species (Rabello *et al.,* 2008; Guo *et al.,* 2009). Most of the induced gene products are either functional proteins or regulatory proteins. The functional proteins include LEA proteins, proteases, water channel proteins, proteins involved in detoxification and enzymes involved in osmolyte biosynthesis, while the regulatory gene products consist of protein kinases, transcription factors, and enzymes involved in the metabolism of abscisic acid and phospholipids. Our study was focused on the expression pattern of genes encoding for functional proteins (Figure 18).

Dehydrin (Dh3)

Dehydrins belong to group II LEA proteins and are highly hydrophilic. Dehydrins usually accumulate at the late stage of embryogenesis, a stage in which the moisture content present in seeds is decreased, and is thus associated with desiccation tolerance in plants (Battaglia *et al.,* 2008; Liu *et al.,* 2017). Although the function of dehydrins is not yet fully understood, several studies have clearly demonstrated their role in tolerance to abiotic stress (Cao *et al.,* 2017; Agarwal *et al.,* 2017).

In the present study, the expression of dehydrin gene (*Dh3)* was relatively quantified during progressive water deficit over five days. At the first time-point, the relative expression of dehydrin was low in all the diploid cultivars and in their respective induced lines compared to the third-time point, however, at the second time point, *Dh3* was strongly expressed in the induced tetraploid ‘Magloire’ with a 1405-fold increase compared to the 17.5-fold increase in its diploid counterpart. The cultivar ‘Varpė’ also showed a similar pattern in which the fold increase in the induced tetraploid (104.1) was significantly higher than the fold increase in its diploid progenitors (60.2). *Dh3* appeared to increase in expression level as the drought period progressed. The highest expression of *Dh3* was observed at the end of the drought stress, with the induced tetraploids showing significantly higher expression than the diploid progenitors, although variations in the cultivars were also observed.

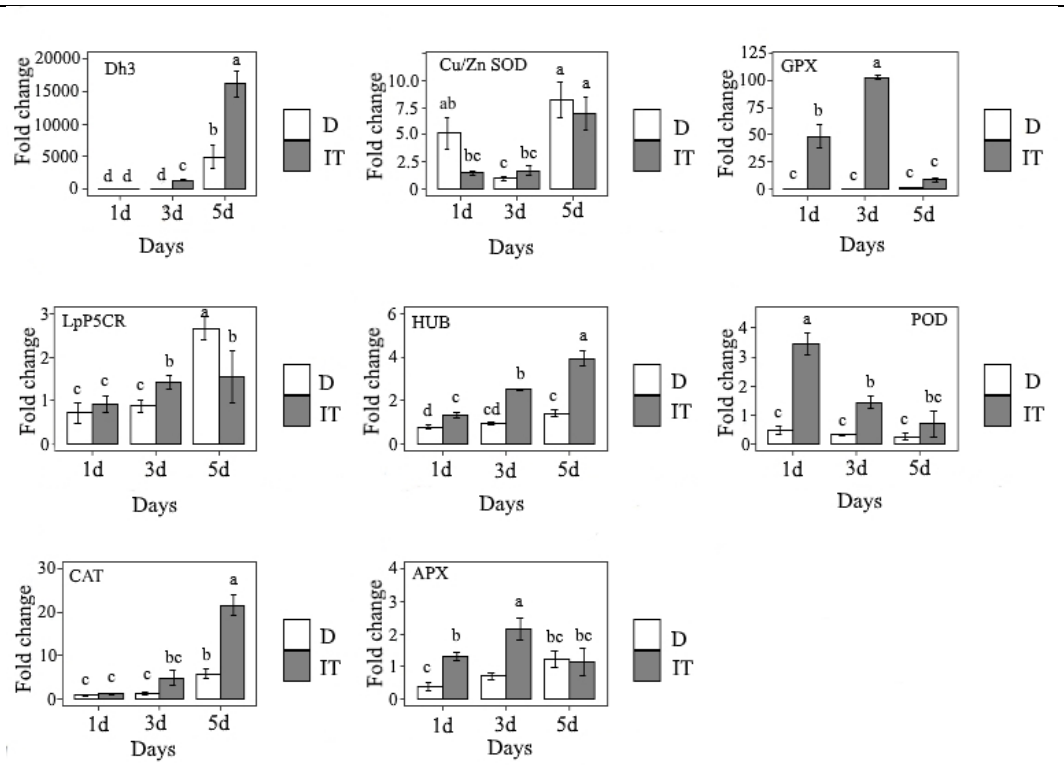
Dehydrins have been found to be mainly involved in stabilising membranes, nucleotides, enzymes and serving as a molecular chaperone to sustain the basic activities of functional proteins of cells under abiotic stress (Eriksson *et al.,* 2011; Graether & Boddington, 2014). Recent studies have revealed that dehydrins can scavenge ROS during stress, and thus increase the ability of plants to tolerate abiotic stress (Liu *et al.,* 2017; Halder *et al.,* 2018).

Relative water content was determined at the end of drought treatment (Table 12). The induced tetraploid lines significantly expressing dh3 genes more than their diploid counterparts also had higher RWC compared to their diploid progenitors. This is similar to the results presented by Park *et al.* (2006), showing a positive correlation between the *Dh3* and *Dh4* transcript accumulation and RWC and drought yield index in a set of Korean barley cultivars. The relative expression of *Dh3* and *Dh9* in barley flag leaf under terminal drought also showed a positive correlation with plant biomass, and chlorophyll a and b contents, and a negative correlation with electrolyte leakage levels and malondialdehyde (Karami *et al.,* 2013). Our result suggests the importance of the *Dh3* gene in the response to drought conditions, and their overexpression could indicate improved tolerance to drought stress, especially in the induced tetraploid lines.

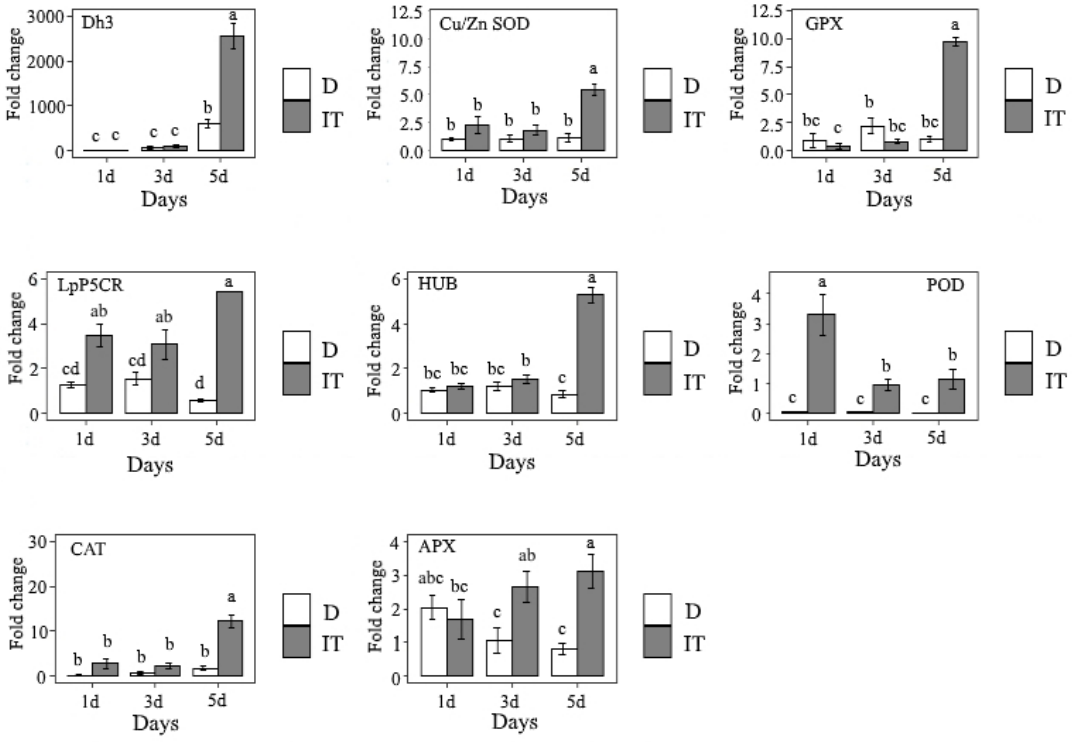
HUB1

Ubiquitin and Ubiquitin-like proteins, collectively known as small ubiquitin-like modifiers (SUMO), have important roles in many cellular and developmental processes in plants (Miura & Hasegawa, 2010), and are part of sophisticated and complex post-translational modification systems (Deribe *et al.,* 2010). Homology to *Ubl (HUB1*), sometimes referred to as *Ubl5*, is a subfamily of the ubiquitin-like proteins structurally similar to ubiquitin, although it shares only 35% amino acid similarities (Downes & Vierstra, 2005). *HUB1* has been identified in plant genomes; however its physiological role in plants is still poorly understood (Downes & Vierstra, 2005). *HUB1* has been associated with many physiological functions in eukaryotes (Benedetti *et al.,* 2006; Mishra *et al.,* 2011).

Plants are able to change the expression pattern of genes when drought is sensed, however, the expression level of these genes depends on factors such as genotypes and differences in ploidy level. Our results showed that diploid cultivars, Westerwolths ryegrass and the corresponding induced tetraploid lines, showed fold increases in *HUB1* expression level when subjected to five days without watering. The induced tetraploid showed higher expression levels than their diploid progenitors however, and the highest *HUB1* expression was observed at the end of the drought stress.



Diploids (D) Magloire and Induced tetraploid (IT) Magloire



Diploids (D) Varpė and induced tetraploid (IT) Varpė

Figure 18. *Candidate gene expression level (fold change) in diploid cultivars 'Magloire' and 'Varpė' and their respective induced tetraploid lines subjected to five days water deficit. Data shown as mean ± standard error of four replicates; the means followed by the same letter within diploids and corresponding induced tetraploid lines are not significantly different (p > 0.05, Duncan's multiple range test)*

The *HUB1* gene function has not been widely studied in plants; only one study has been carried out on the function of this gene in plants. Patel *et al.* (2015) showed that the accumulation of *HUB1* transcript increased in perennial ryegrass exposed to water stress, and was repressed when water was restored, suggesting that the *HUB1* gene plays a role in drought tolerance. Our results showed a *HUB1* gene expression fold increase of approximately 1–2 fold in the diploid cultivars compared to the 4–5 fold increase in the induced tetraploids. Patel *et al.* (2015) clearly demonstrated that the transgenic lines of *Lolium perenne* overexpressing the *LpHUB1* gene had a 2–3 fold increase in the endogenous transcript. The overexpressing transgenic lines of *Lolium perenne* demonstrated higher relative water content, increased chlorophyll content and possibly an enhanced rate of photosynthesis, which are all indicators of stress tolerance.

Table 12. *The relative water content (RWC) of diploid cultivars and induced tetraploid lines subjected to a five day water deficit with their respective controls*

Cultivars/ induced lines	RWC
Magloire 2x control	93.54 ± 0.79 a
Magloire-4x control	94.33 ± 1.24 a
Magloire 2x	73.27 ± 1.52 c
Magloire-4x	80.71 ± 1.27 b
Varpė 2x control	93.61 ± 2.24 a
Varpė 4x control	93.69 ± 0.61 a
Varpė 2x	68.10 ± 0.61 d
Varpė 4x	74.20 ± 1.12 c

Note. Data shown as mean ± standard error of three replicates; the means followed by the same letter within each column are not significantly different ($p > 0.05$, Duncan's multiple range tests)

Superoxide dismutase (Cu/Zn SOD), catalase (CAT), ascorbate perioxidase (APX), guaiacol peroxidase (POD), and glutathione peroxidase (GPX)

When plants are subjected to drought, the ROS generation such as H_2O_2 (hydrogen peroxide), O_2^- (superoxide) and OH (hydroxyl) radicals increases. Excess ROS production can damage cellular structures, proteins, lipids and nucleic acids, which can ultimately lead to cell death (Uzilday *et al.,* 2012). Previous studies have indicated that enhanced antioxidant enzymes activity improved the tolerance to drought stress in many plant species, such as rice and cotton (Basu *et al.,* 2010; Deeba *et al.,* 2012). The genes involved in the antioxidative metabolic pathways are activated during drought stress and the gene products are vital in detoxifying ROS. Superoxide dismutase is usually the first line of defence against ROS (Liu & Huang, 2000). SOD facilitates the dismutation of O_2

to H_2O_2 and O_2. The H_2O_2 generated is still toxic to the cells and enzymes such as CAT, POD, APX, and GPX helps to convert H_2O_2 to water.

Our study indicated an increase in the expression level of Cu/Zn SOD, especially at the end of the drought treatment. Variations in expression pattern from the first time-point to the third time-point were also observed. Both the diploid and induced tetraploid lines of 'Magloire' showed a significant increase in expression level compared to the cultivar 'Varpė'. The diploid cultivar 'Magloire showed a higher expression level than its induced tetraploid counterpart, but this observation was quite the opposite in the 'Varpė' cultivar, with the induced tetraploid showing a higher expression level of Cu/Zn SOD. The genotypic difference could be one of the reasons the expression pattern of the gene varied. In general, the increase in the expression level of Cu/Zn SOD could be due to an increase in the production of active oxygen species, and is consistent with results from other studies reporting an increase in the expression of Cu/Zn SOD during drought conditions (Liu & Huang, 2000). The overexpression of Cu/Zn SOD in the transgenic lines of *Nicotiana tabacum* also improved the chloroplast antioxidant system and hence improved their tolerance to drought and osmotic stress compared to the wild type (Hamid Badawi *et al.,* 2004).

Cool-season Westerwolths ryegrass generally requires a relatively large amount of water to sustain growth. When plants are subjected to drought conditions, one of their first responses is the closure of stomata to reduce water loss from transpiration. When photosynthesis proceeds with closed stomata, there is depletion in the concentration of intracellular CO_2 due to an increased gas diffusion barrier. Westerwolths ryegrass is a C3 plant prone to photorespiration during drought, and higher temperatures than C4 plants, which are capable of minimising photorespiration by separating the initial CO_2 fixation and the Calvin cycle in space. Photorespiration usually leads to the production of H_2O_2 in the peroxisomes.

CAT, APX, POD and GPX are all capable of reducing H_2O_2 to water and O_2. Many studies have shown that CAT, APX and GPX play the most important roles in ROS scavenging (Becana *et al.,* 2000; Caverzan *et al.,* 2012; Uarrota *et al.,* 2016). The overexpression of these genes has been suggested to confer tolerance to drought on plants. In this study, the expressions of these genes were upregulated during drought stress. The expression of the genes also varied between cultivars, and also across the duration of the stress. Generally, the induced tetraploids showed significantly higher expression levels of genes involved in antioxidation compared to their diploid progenitors.

LpP5CR

When undergoing water stress, many plants adjust their osmotic potential in the cells through the accumulation of osmoprotectants such as proline. Reports have indicated an increase in the synthesis of proline as well a reduction in the catabolism of proline under abiotic stress (Verbruggen & Hermans, 2008; Kavi Kishor *et al.*, 2015).

Δ1-pyrroline-5-carboxylate synthetase (P5CS) and Δ1-pyrroline-5-carboxylate reductase (P5CR) are the major enzymes involved in the biosynthesis of proline in plants. P5CR catalyses the conversion of Δ1-pyrroline-5-carboxylate to proline.

Our results indicated an increase in the expression level of *LpP5CR*. Genotypic variations were also observed in the expression levels of this gene, with the induced tetraploid line 'Varpė' showing the highest expression level at the end of the drought stress. Pyrroline-5-carboxylate reductase has been identified as not catalysing the rate-limiting step in proline biosynthesis. While some studies have indicated that the overexpression of *P5CR* gene does not correlate with an increase in proline accumulation (Szoke *et al.*, 1992), another study with *Lolium perenne* indicated changes in the transcript of *LpP5CR* gene in response to various abiotic stress, thus suggesting that the gene may yet play an important role in proline accumulation (Li *et al.*, 2015).

The gene expression pattern in the induced tetraploid of Westerwolths ryegrass differs from that of their diploid counterparts, and some of the expression changes might be a result of the increase in the copy number of the chromosomes. This expression change could affect all the genes equally, resulting in their uniformly increased expression level (Livak & Schmittgen, 2001). Autopolyploidy can affect the physiological, cytological, biochemical and genetic process in various organisms (Sharma & Gohil, 2013; del Pozo & Ramirez-Parra, 2015; Lloyd & Bomblies, 2016).

In conclusion, the abundant supply of both diploid and tetraploid forage grass cultivars on the market makes it difficult for farmers to make an informed decision. Breeders, on the other hand, are faced with the never-ending challenge of steering their breeding programs to meet the challenges that might arise in the future. Current climatic trends and future models suggest an increase in the areas facing constant or recurrent droughts, making breeding for drought-tolerant forage cultivars a top priority. Understanding the differential gene expression patterns of diploid and induced tetraploid lines of Westerwolths ryegrass to abiotic stress could help to improve the germplasm, and result in successful agronomic production in sub-optimal climatic conditions.

CONCLUSIONS

1. The induction of tetraploids from diploid cultivars of Westerwolths ryegrass was achieved using both colchicine and APM, however, the efficiency of the mitosis inhibitors depends on the optimal concentration, exposure time, affinity and toxicity of the inhibitors. A concentration of 10 mM and 8 mM with an exposure time of three and four hours respectively were found to be most efficient in inducing tetraploids from diploid cultivars.
2. An increase in ploidy confer advantages in the performance of the induced tetraploid lines in the field trials, as observed in the dry matter yield and morphological traits, especially in water deficit conditions, compared to diploid progenitors.
3. Salinity stress significantly inhibits the germination of Westerwolths ryegrass seeds. Chromosome duplication appears to play an important role during germination in saline conditions. The induced tetraploid lines in most cases had a higher germination index and lower T50 values than their diploid progenitors. At the vegetative stage, the induced tetraploid lines had higher antiradical activity during salinity stress than their parental diploids, indicating that polyploidy contributes to a tolerance to salinity that often leads to oxidative injury in plant cells.
4. The induced tetraploid lines of Westerwolths ryegrass had higher antiradical activity and phenolic content during mild drought periods than their parental diploids. There was also a strong correlation between antiradical activity and drought recovery, indicating that polyploidy contributes to tolerance to drought.
5. The expression pattern of drought related genes differs in the diploid cultivars and induced tetraploid lines. The induced tetraploid lines showed higher expression level genes encoding for functional proteins, which gives them an advantage over the diploid progenitors in response to drought.

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LIST OF PUBLICATIONS

Articles in journals with impact factor in *Clarivate Analytics Web of Science* database

1. **Akinroluyo, O.**, Jaškūnė, K., Kemešytė, V., Statkevičiūtė, G. 2019. Differences in salt tolerance between diploid and autotetraploid of *Lolium multiflorum* at the germination and vegetative stages. Zemdirbyste-Agriculture 106 (4): 329–336. DOI 10.13080/z-a.2019.106.042.

2. **Akinroluyo, O.**, Jaškūnė, K., Kemešytė, V., Statkevičiūtė, G. 2019. Drought stress response of Westerwolths ryegrass (*Lolium multiflorum* spp. *multiflorum*) cultivars differing in their ploidy level. Zemdirbyste-Agriculture: Accepted.

Book Chapter

1. **Akinroluyo O**., Statkevičiūtė G., Kemešytė V. 2018. Tetraploid Induction in *Lolium multiflorum*. Brazauskas G. et al. (eds). Breeding grasses and protein crops in the era of genomics. Springer, p. 73–77. DOI 10.1007/978-3-319-89578-9_13.

Conferences attended

1. **Akinroluyo O.,** Kemešytė, V., Statkevičiūtė G. Tetraploid induction in Annual ryegrass. The International conference of young scientists “Young Scientists for Advance in Agriculture” 10 November 2016, Vilnius, Lithuania. Oral presentation.
2. **Akinroluyo O.,** Kemešytė, V., Statkevičiūtė G. Tetraploid induction in *Lolium multiflorum.* The joint meeting of EUCARPIA Fodder Crops and Amenity Grasses section “Breeding Grasses and Protein Crops in the Era of Genomics” September 11–14, 2017. Vilnius, Lithuania. Oral presentation.
3. **Akinroluyo O.,** Kemešytė, V., Statkevičiūtė G. Effect of ploidy level on drought stress response in annual ryegrass. VII Baltic Genetics Congress. October 24–27. Riga, Latvia. Pp 191. Oral presentation.
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Differences in salt tolerance between diploid and autotetraploid lines of *Lolium multiflorum* at the germination and vegetative stages

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Abstract

Soil salinity is a global challenge emanating from climatic changes, depletion of fresh water reserves and extensive irrigation practices among other factors. Soil salinization still remains a huge concern in the realization of sustainable agricultural production. While emphasis has been placed on the food crops, forage production, which is an important component of the food chain, is affected as well.

The aim of this study was to evaluate the morphological and physiological response to salinity stress in diploid cultivars and auto-induced tetraploid lines of annual ryegrass (*Lolium multiflorum* spp. *multiflorum*). Diploid seeds and their induced tetraploid counterparts were germinated on filter paper moistened with different concentrations of sodium chloride (NaCl) solutions, and seedlings were treated with 500 mM NaCl for 10 days in controlled conditions. The effect of different salt concentrations on germination and seedlings was studied. Results showed that seeds from the induced tetraploid lines despite being bigger had higher germination index and lower median germination time (T50) values compared to the diploid progenitors. At the seedling stage, increase in the ploidy level had a role in conferring improved tolerance to salinity stress. The induced tetraploid lines had an advantage over their diploid counterparts as the induced tetraploid lines had significant reduction in their growth in response to salinity stress, higher relative water content and antioxidant activities.

Key words: abiotic stress, annual ryegrass, antioxidant activity, ploidy level.

Introduction

Environmental stresses affect the productivity of many agricultural crops globally. The world population is constantly increasing and is estimated to reach 9 billion people by the year 2050; however, the land acreage is fairly constant, therefore there is a need to explore lands that have not been used for agricultural purposes such as semi-arid regions and forests and high salinized soils to improve food production globally (Godfray et al., 2010).

Drought and salinity are one of the two main abiotic stresses that affect plant productivity. Recently, the threat posed by global warming is gradually becoming a reality as extended periods of high temperatures were recorded in the northern parts of Europe in 2018 (UNCC, 2019). Also, more than 6% of the global land area has severe salinity problem and this translates to about 800 million hectares (Yadav et al., 2011). In addition, extreme drought requires irrigation farming practices, which ultimately can result in increased soil salinity over a period of time (Qadir et al., 2014). A good approach to food sustainability is to increase the crop productivity in these suboptimal soil conditions, especially in high salinized soils.

High soil salinity affects the uptake of water from the roots and also cell growth and metabolism in the roots. Also, high saline conditions in the soil often lead to structural defects, high root zone pH, oxygen deficiency, impaired root respiration as well as nutritional imbalances (Roy et al., 2014). Also, studies have shown that plants grown on high saline substrates express salt-specific stress, oxidative stress and osmotic and ionic stress (Muscolo et al., 2013). The ionic stress is manifested by the accumulation of sodium and chloride ions in the plant tissues. The uptake of these ions causes severe ionic imbalance as the high concentration of sodium ions inhibits the uptake of potassium ions (Assaha et al., 2017). The unavailability of potassium ions results in physiological impairment in growth and development of the plants and can result in plant death. High soil salinity also often results in hyperosmotic stress, where there is a progressive loss of water from the leaves, while the absorption of water by the roots is significantly reduced (Tang et al., 2015). This hyperosmotic stress triggers physiological changes that are detrimental to the development of the plant including a reduction in photosynthetic activities and an increase in the

reactive oxygen species (ROS) produced (Acosta-Motos et al., 2017). The ROS emanating from oxidative stress caused by high salinity substrate damages the cellular components and disrupts important cellular functions.

Annual ryegrass (*Lolium multiflorum* spp. *multiflorum*) is an important forage grass species from the family Poaceae and is widely cultivated in temperate regions. It is also widely used as a catch crop and is efficient in preventing soil erosion (Humphreys et al., 2010). It occurs naturally as diploid (2n = 2x = 14). Chromosome duplication has been achieved in many plant species and has been found to increase their tolerance to environmental stresses compared to their diploid counterparts (Sattler et al., 2016), while other studies reported diploids to have superior tolerance to abiotic stresses (Helgadóttir et al., 2018). However, little is known on the tolerance to salinity stress between diploid and tetraploid lines of annual ryegrass.

The aim of this study is to evaluate the role that ploidy has in the tolerance to salinity stress in annual ryegrass. Tetraploid lines were induced from diploid cultivars to maintain their genetic homogeneity and thus reducing the effect of genetic differences. The physiological response and the antioxidant activity changes to salinity stress were compared between diploid cultivars and auto-tetraploid lines.

Materials and methods

The research was conducted in 2019 at Laboratory of Genetics and Physiology, Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry.

Plant material and chromosome doubling. Tetraploid lines were induced from 8 diploid cultivars (Table 1) of annual ryegrass as described in Akinroluyo et al. (2018). The induced tetraploid lines were grown to maturity; seeds were collected and sown for the second generation. The ploidy level was also evaluated for the second generation of induced tetraploid lines; seeds of true tetraploid plants were collected and used in this study. In addition to the induced tetraploids, tetraploid cultivars 'Peleton', 'Caremo' and 'Wesley' were also used in this study.

Table 1. List of cultivars and induced tetraploid lines of *Lolium multiflorum* spp. *multiflorum* used in this study

Cultivar	Ploidy	Origin	Name of induced tetraploid
Druva	2×	Latvia	Druva-4x
Varpė	2×	Lithuania	Varpė-4x
Magloire	2×	France	Magloire-4x
Prompt	2×	France	Prompt-4x
Top speed	2×	France	Top speed-4x
Surrey nova	2×	USA	Surrey nova-4x
Grazer	2×	Germany	Grazer-4x
Shoot	2×	Denmark	Shoot-4x
Peleton	4×	Denmark	
Wesley	4×	Denmark	
Caremo	4×	Denmark	

Seed germination. Fifteen seeds of each cultivar and induced tetraploid line were placed on three layers of filter paper in a Petri dish in three replicates. The filter paper was moistened with either distilled water or different concentrations of sodium chloride (NaCl) solution. The salinity concentrations ranged from 120 to 200 mM. The germination was recorded daily for 10 days. The experiment was repeated three times. The germination percentage (GP), germination index (GI), mean germination time (MGT) and the median germination time (T50), which is the time to reach 50% of the germination in all the seeds, values were calculated as described in Coolbear et al. (1984) and Kader (2005) and modified by Farooq et al. (2005):

$GP = 100 (x / n)$,

where x is the total number of germinated seeds, n – the total number of seeds;

$GI = (10 \times n1) + (9 \times n2) + \cdots + (1 \times n10)$,

where n1, n2 ... n10 represents the germinated seed on the first, second and subsequently till he 10^{th} day; 10, 9 ... 1 are weights given to the number of germinated seeds on the first, second and subsequent days till the 10^{th} day, respectively;

$MGT = \sum nt / \sum n$,

where t (days) represents the time from the beginning of germination test, n – the number of germinated seeds at time t;

$$T50 = \frac{ti + [\{(N/2) - ni\}(ti - tj)]}{ni - nj},$$

where N represents the final number of germination, n_i and n_j are cumulative number of seeds germinated by adjacent counts at times t_i and t_j when $n_i < N/2 < n_j$.

Effect of salt stress on annual ryegrass seedlings. Seeds from diploid, tetraploid and induced tetraploid lines and cultivars of annual ryegrass were germinated on 50:50 perlite:vermiculite mix substrate (vol.) in round plastic pots (diameter 9 cm, height 8 cm). The plantlets were allowed to develop for three weeks at 25 ± 2°C with a

16/8 h light/dark photoperiod before inducing the salinity stress. The plantlets were treated with 500 mM NaCl for 10 days. New unfolding leaves were marked at the nodes on the first day in five different plants in separate pots, and the leaf elongation was recorded on a daily basis at exactly the same time. The treatment was done in three replicates.

Relative water content (RWC) measurement. Plant water status at the end of the stress was expressed in terms of the RWC. Fresh leaf samples were cut and weighed immediately to determine the fresh weight (FW). The leaves were then placed in plastic bag containing water and left for 6 hours to reach the turgid weight (TW). Then the leaves were blotted dry and the turgid weight was determined. Finally the leaves were placed in an oven at 70°C for 48 hours to determine the dry weight (DW). The relative water content was calculated using the formula (Smart, Bingham, 1974): RWC% = 100[(FW − DW) / (TW − DW)].

Antioxidant activity measurement. The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was measured by slightly modifying the method described by Brand-Williams et al. (1995). The plant material was prepared by drying, homogenizing, suspending 0.5 g of stressed leaf samples and their respective control in 70% methanol, followed by extraction in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH & Co. KG, Germany) for 60 min at 50°C and 480 W. Two ml of DDPH solution in 70% v:v methanol was mixed with 2 μL leaf methanol extract. The reduction in absorbance at 515 nm was measured and expressed as Trolox equivalent (TE) antioxidant capacity.

Statistical analysis. The statistical analysis was carried out using software *SAS*, version 9.4 (SAS Institute Inc., USA). The analysis of variance (ANOVA) was carried out and the significant differences between the means were determined using the Duncan's multiple range test. Correlation analysis was also carried out to check for relationships among the traits.

Results

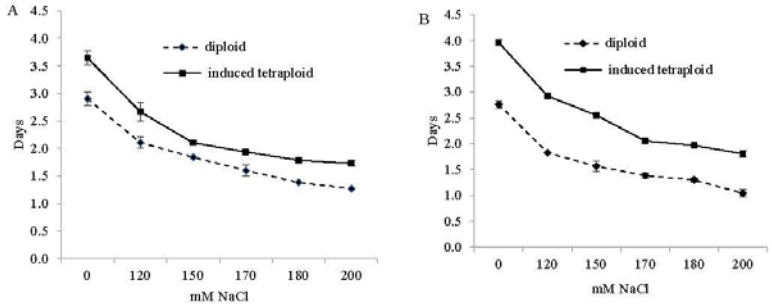
Genome duplication effect on germination of annual ryegrass under salt stress. Seeds from both cytotypes varied in length and weight. The induced tetraploid seeds were longer and heavier than their diploid progenitors (Table 2).

Table 2. The seed length and weight of diploid cultivars and respective induced tetraploid lines of *Lolium multiflorum* spp. *multiflorum*

Diploid cultivar / induced tetraploid line	Seed length mm	1000 seed weight g
Magloire	5.83 ± 0.17	2.77 ± 0.07
Magloire-4×	7.17 ± 0.17*	4.03 ± 0.05*
Druva	6.00 ± 0.00	2.39 ± 0.06
Druva-4×	7.00 ± 0.29*	4.23 ± 0.11*
Varpė	5.50 ± 0.00	2.81 ± 0.10
Varpė-4×	8.33 ± 0.61*	4.95 ± 0.06*
Grazer	5.00 ± 0.29	2.81 ± 0.08
Grazer-4×	6.50 ± 0.51*	4.60 ± 0.08*
Prompt	5.17 ± 0.17	2.70 ± 0.09
Prompt-4×	6.17 ± 0.17*	3.78 ± 0.05*
Shoot	5.67 ± 0.17	2.19 ± 0.08
Shoot-4×	7.33 ± 0.34*	4.08 ± 0.11*
Surrey nova	4.83 ± 0.17	2.93 ± 0.10
Surrey nova-4×	6.67 ± 0.17*	3.89 ± 0.08*

Note. Data shown as mean ± standard error of three replicates; the means followed by * between diploid cultivar and corresponding induced tetraploid line are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

The effect of salinity stress on germination is shown in Figure 1 and Table 3. Salinity stress appears to delay the outset of germination or inhibit germination in both cytotypes and across the cultivars. Also, the inhibition of germination is increased as the salinity concentration increased ($r = 0.86$, $p \leq 0.01$).



Note. The error bars represent the standard error of the mean.

Figure 1. Germination index of diploid cultivars and respective induced tetraploid lines 'Magloire' (A) and 'Varpė' (B) of *Lolium multiflorum* spp. *multiflorum* in different NaCl concentrations

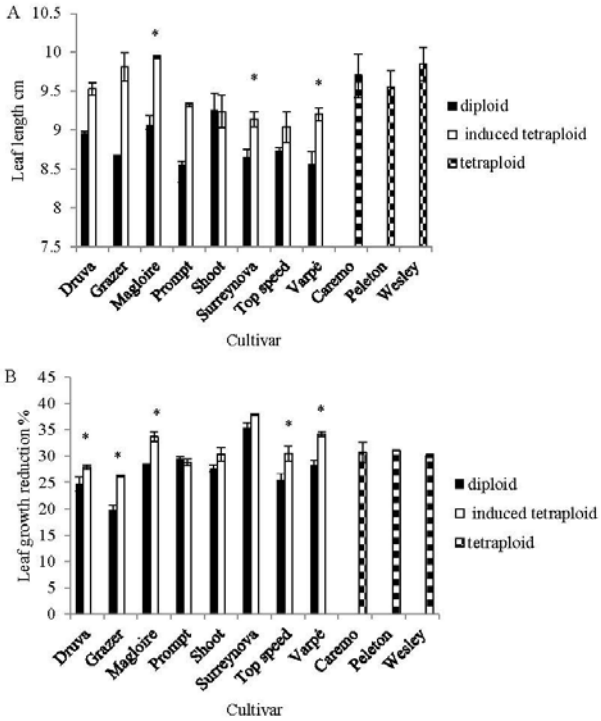
Table 3. Germination percentage, mean germination time, germination index and median germination time (T50) of different diploid cultivars and the respective induced tetraploids of *Lolium multiflorum* spp. *multiflorum* after salinity (200 mM NaCl) treatment

Diploid cultivar / induced tetraploid line	Germination %	Mean germination time, days	Germination index, days	Median germination time (T50), days
Magloire (control)	91.67 ± 2.33 abc	4.50 ± 0.00 fg	2.93 ± 0.22 cd	3.93 ± 0.07 fghi
Magloire-4× (control)	93.33 ± 0.00 abc	3.93 ± 0.07 h	3.62 ± 0.09 ab	3.45 ± 0.05 i
Magloire	63.33 ± 3.33 de	8.00 ± 0.09 bc	1.29 ± 0.04 gh	7.50 ± 0.00 ab
Magloire-4×	90.64 ± 3.33 abc	7.48 ± 0.05 c	1.87 ± 0.09 f	6.58 ± 0.25 c
Druva (control)	91.67 ± 2.33 abc	4.33 ± 0.00 gh	3.18 ± 0.38 bc	3.74 ± 0.01 ghi
Druva-4× (control)	98.00 ± 3.33 ab	4.38 ± 0.02 gh	3.37 ± 0.11 bc	3.85 ± 0.08 fghi
Druva	46.67 ± 0.00 ef	6.64 ± 0.07 d	1.10 ± 0.00 hi	7.58 ± 0.04 ab
Druva-4×	53.33 ± 0.00 ef	6.88 ± 0.13 d	1.18 ± 0.01 h	6.33 ± 0.04 cd
Varpė (control)	80.00 ± 0.00 bcd	4.75 ± 0.09 fg	2.56 ± 0.04 de	4.31 ± 0.06 efg
Varpė-4× (control)	100.00 ± 0.00 a	3.90 ± 0.04 h	3.88 ± 0.04 a	3.44 ± 0.02 i
Varpė	46.67 ± 6.66 ef	8.23 ± 0.11 ab	0.88 ± 0.14 hi	7.88 ± 0.38 ab
Varpė-4×	84.25 ± 3.47 abc	6.83 ± 0.08 d	1.71 ± 0.09 fg	6.25 ± 0.05 cd
Grazer (control)	82.30 ± 5.87 abc	5.35 ± 0.44 e	2.40 ± 0.10 e	4.58 ± 0.20 e
Grazer-4× (control)	95.67 ± 3.33 ab	4.93 ± 0.14 ef	3.01 ± 0.03 cd	4.43 ± 0.15 ef
Grazer	38.42 ± 2.34 f	7.88 ± 0.28 bc	0.70 ± 0.04 i	7.52 ± 0.15 ab
Grazer-4×	65.33 ± 2.34 de	6.73 ± 0.21 d	1.21 ± 0.03 h	6.24 ± 0.08 cd
Prompt (control)	98.00 ± 3.33 ab	4.25 ± 0.11 gh	3.53 ± 0.27 ab	3.64 ± 0.06 hi
Prompt-4× (control)	100.00 ± 0.00 a	4.50 ± 0.10 fg	3.40 ± 0.06 bc	3.84 ± 0.09 fghi
Prompt	78.37 ± 5.87 cd	6.58 ± 0.18 d	1.81 ± 0.19 f	6.08 ± 0.42 cd
Prompt-4×	56.45 ± 5.93 e	8.04 ± 0.24 b	1.09 ± 0.23 hi	7.42 ± 0.09 ab

Note. Data shown as mean ± standard error of three replicates; the means followed by the same letter within each column are not significantly different ($p > 0.05$, Duncan's multiple range test).

The germination percentage, mean germination time, germination index and T50 were calculated for both cytotypes. The result showed that overall germination parameters were reduced in the seeds of both diploid cultivars and the induced tetraploids when compared with their respective control; however, chromosome duplication appears to have a role in improving the tolerance to salinity stress at the germination stage as seen in the germination index and T50 values. The cultivar differences also contributed to the observed differential response to the salinity treatments.

Genome duplication effect on the seedling growth of annual ryegrass under high saline conditions. Seedlings of both diploid and tetraploid *Lolium multiflorum* spp. *multiflorum* (three weeks old) were grown in controlled conditions. New unfolding leaves were measured in this experiment. The leaf elongation was determined during stress treatment as well as in the control in non-destructive daily leaf length measurements. The leaf elongation was measured at the onset of the stress till the end of the experiment. The effect of high salinity treatment was apparent in both induced tetraploids and the diploid cultivars showing a significant reduction in the leaf growth and shoot development. Our results showed that the induced tetraploids had longer leaf when compared to their parental diploids during the salinity treatment (Fig. 2A). Also, the induced tetraploids showed a higher reduction in their leaf elongation than their diploid progenitors when compared with their respective control experiment (Fig. 2B). Differences in the cultivar leaf length and leaf growth reduction were also observed.



Note. Data shown as mean ± standard error of three replicates; the means followed by * between diploids and corresponding induced tetraploids are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Figure 2. The leaf length (A) and leaf growth reduction (B) in seedlings of *Lolium multiflorum* spp. *multiflorum* treated with 500 mM NaCl

Plants subjected to salinity stress showed signs of wilting when compared to the control. Salinity stress also reduced the relative water content. The relative water content varied across the cultivars and most of the induced tetraploids were found to have higher relative water content when compared to their diploid progenitors (Table 4). The differences observed between the means were significant.

Table 4. The effect of salinity stress on the relative water content and the antioxidant activity response in diploid cultivars and the respective induced tetraploid lines of *Lolium multiflorum* spp. *multiflorum* seedlings

Diploid cultivar / induced tetraploid line	Relative water content %	Antioxidant activity (control) µmol TE g^{-1}	Antioxidant activity (500 mM NaCl) µmol TE g^{-1}
Varpė	68.76 ± 0.19 g	23.73 ± 3.22 de	34.97 ± 0.78 fg
Varpė-4×	75.87 ± 0.49 de	35.48 ± 1.34 a	47.26 ± 0.95 ab
Druva	80.18 ± 1.18 c	32.71 ± 1.01 ab	36.63 ± 1.35 g
Druva-4×	71.77 ± 0.5 f	29.55 ± 1.35 bc	44.26 ± 0.98 bc
Magloire	69.06 ± 0.31 g	33.42 ± 1.81 ab	41.51 ± 2.63 cd
Magloire-4×	80.64 ± 1.01 c	33.18 ± 1.15 ab	49.15 ± 0.11 a
Grazer	75.92 ± 1.43 de	22.97 ± 0.15 e	28.82 ± 2.28 h
Grazer-4×	74.37 ± 0.93 ef	24.73 ± 0.55 de	39.00 ± 1.97 de
Surrey Nova	87.61 ± 1.02 a	27.34 ± 0.54 cde	32.84 ± 1.34 fg
Surrey Nova-4×	83.50 ± 1.01 b	23.33 ± 2.87 de	42.70 ± 2.33 c
Prompt	73.67 ± 0.21 ef	27.82 ± 0.72 cd	36.52 ± 1.77 ef
Prompt-4×	81.00 ± 1.01 bc	28.14 ± 0.02 cd	38.84 ± 2.35 de
Top speed	77.36 ± 0.69 d	27.46 ± 0.50 cde	35.34 ± 1.45 efg
Top speed-4×	88.08 ± 0.63 a	24.01 ± 0.78 de	41.34 ± 1.23 cd
Shoot	74.02 ± 1.00 ef	30.02 ± 0.52 bc	35.66 ± 0.55 efg
Shoot-4×	80.42 ± 0.36 c	32.81 ± 1.14 ab	43.24 ± 0.89 c

Note. Data shown as mean ± standard error of three replicates; the means followed by the same letter within each column are not significantly different ($p > 0.05$, Duncan's multiple range test); TE – Trolox equivalent.

Genome duplication effect on the antioxidant activities of annual ryegrass under salt stress. The antioxidant activities in response to the salinity stress were determined in both diploids and tetraploids. The result showed that the induced tetraploids produced more antioxidant activity response than their diploid progenitors, except for the cultivar 'Prompt'. A significant positive correlation was also found between the antioxidant activities and the germination index ($r = 0.55$, $p \leq 0.05$) and the reduction in leaf elongation during salinity stress ($r = 0.64$, $p \leq 0.05$).

Discussion

The outset of the germination process begins with seed imbibition, in which water is absorbed by seeds to make the nutrients in the endosperm available. Imbibition is a critical stage in the germination process and has to be completed before germination occurs. The imbibition process depends on several factors, including the temperature, water, oxygen, permeability of the seed coat, seed size and osmotic potential (Louf et al., 2018). Several studies (Matthews, Khajeh-Hosseini, 2007; Ahmed et al., 2017) have shown that the germination and seedling growth are significantly affected by salt stress; however, little is known on the role that ploidy has in annual ryegrass. Studies have reported that imbibition is completed faster in the smaller seeds within the same ploidy level because of the increase in surface area to volume ratio, hence, increasing the absorption of water is leading to faster germination (Schneider, 1998; Souza, Fagundes, 2014). However, other studies reported that tetraploid seed germinated faster than their diploid counterparts (Eliášová, Münzbergová, 2014).

Our results showed that induced tetraploid seeds of annual ryegrass were bigger and heavier than their parental diploids and in most cases germinated faster than the diploid counterparts. This raises the question if increase in the ploidy level confers an advantage in seed germination and, even more, under stress condition.

The performance of seeds can be seen by comparing the germination parameters between the diploids and induced tetraploids. The mean germination time (MGT) for both cytotypes varied across the cultivars and induced lines and was clearly not accurate in explaining the role of ploidy in seed germination. Although some researchers have used the MGT to evaluate the seed vigour of many plants (Matthews et al., 2012; Chen et al., 2013), other studies found the MGT to be inaccurate arguing that seeds can have different final germination percentage and have the same MGT because seeds can germinate across a different spread (Kader, 2005). The MGT expressed better the day, in which most of the seeds germinated in a seed lot or accurately defined the mean lag period between the start of imbibition and germination for each seed (Matthews, Khajeh-Hosseini, 2007). On the other hand, the T50 gave a better understanding of the speed of germination (Soltani et al., 2015). Our results indicated that the induced tetraploids had lower T50 values, especially during the salinity stress, except for the cultivar 'Prompt'.

The germination index gives a more accurate measurement of the germination than the germination percentage and the mean germination time because it takes cognizance of the germination percentage, speed of germination and the spread of germination (Javaid et al., 2018). It is clearly seen from our results (Table 3) that the induced tetraploid seeds had a higher germination index than their diploid progenitors both in the control and under stress conditions, except for the cultivars 'Prompt' and 'Druva'. In diploid cultivar 'Prompt' the seeds had a higher germination index than the induced tetraploid counterpart only during salinity stress, while no significant difference was found in the germination index of diploid and induced tetraploid seeds of 'Druva' both in the control and under salinity stress.

Generally, the effect of salinity on seed germination occurs via the ionic toxicity, osmotic effect or the combination of both effects (Panuccio et al., 2014). Salinity often increases the osmotic potential while decreasing the water potential making water unavailable to plants. The lower the osmotic potential in seeds, the more seed can absorb water and complete imbibition. Zhang et al. (2010) further explained that seeds in saline conditions can have a decreased osmotic potential by excluding salt from the cells while using other organic solutes as osmolites to maintain the osmotic potential. Bigger seeds at a higher ploidy level are at an advantage here than smaller seeds at a lower ploidy level, as they have more carbon reserves and can generate a lower osmotic potential and thus, alleviating the need to absorb sodium. Alternatively, seeds can accumulate and use sodium and chloride ions as osmolites while having a mechanism that neutralizes their toxic effect. However, it remains unclear what is the mechanism used by annual ryegrass in maintaining a water potential gradient during the salinity.

Our results showed a medium positive ($r = 0.55$) correlation between antioxidant activities during salinity stress at the seedling stage and the germination index. Interestingly, the increases in antioxidant activities during salinity stress at the seedling stage in the induced tetraploid lines were significant when compared to their parental diploid, except in the cultivar 'Prompt', which also had higher germination index compared to its corresponding induced tetraploid. While some studies have used salinity tolerance at the germination and seedling stages as an indicator for screening tolerant genotypes (Shahid et al., 2012; Ravelombola et al., 2017), other studies indicated the opposite suggesting that the tolerance to salinity stress might be specific for various developmental stages (Lauchli, Epstein, 1990). However, the relationship between the antioxidant activities at the seedling stage and the germination index alone cannot fully explain if the tolerance at the germination stage in *Lolium* could be an

indicator of tolerance at other developmental stages. More studies involving physiological responses at various developmental stages are needed to understand how seeds reduce their osmotic potential in the germination process during salinity stress and also to determine an effective tolerance screening stage in *Lolium*.

At the vegetative stage, the first effect of salinity stress occurs in the root system of plants and this impairs the growth due to the osmotic stress. The osmotic stress reduces the availability of water to the plants and also generates reactive oxygen species (ROS) (Ashraf, Foolad, 2013). One of the first metabolic responses of plants under stress is the growth inhibition and down regulation of energy metabolism indicating that plants conserve energy (Cramer et al., 2011). The reduction in growth especially in the leaf area usually occurs by inhibiting protein synthesis. This is an avoidance mechanism that helps to reduce water loss via transpiration (Rodriguez et al., 2005). Our results show that while the induced tetraploids have longer leaves than their diploid counterparts, the induced tetraploids were able to slow down the leaf growth more than their diploid progenitors. Also, the induced tetraploid lines in most cases had higher relative water content when compared to their corresponding diploid progenitors. The response to salinity stress involves a cascade of reactions involving many genes at the molecular level; however, duplication of the genetic materials seems to have an advantage over the diploid in the first response to salinity in annual ryegrass.

Prolonged exposure to salinity stress leads to ion toxicity and nutrient imbalance. This often results in sodium toxicity and generation of ROS (Torre-González et al., 2017). Plants growing in optimal conditions are said to be redox homeostatic because there is equilibrium in the production and scavenging of ROS. When plants generate high levels of ROS with an inefficient mechanism in scavenging the ROS, it causes an imbalance in the cellular redox, hence leads to oxidative stress (Sharma et al., 2012). Plants generally combat high levels of ROS by activating the enzymatic and non-enzymatic system that scavenges the ROS. The enzymatic system involves enzymes such as superoxide dismutase, peroxidase, catalase, glutathione reductase and ascorbate peroxidase (Huseynova et al., 2014). The non-enzymatic system involves tocopherols, glutathione and ascorbic acid, which are also involved in ROS detoxification (Caverzan et al., 2016).

The synthesis of the antioxidants and their activities are altered when plants are subjected to stress conditions. In addition, polyploids can increase their tolerance to stress by altering their physiology, phenology and morphology (Adams, Wendel, 2005). Reports have shown that the increase in stress tolerance correlates with the increase in antioxidant activities (Aghaei et al., 2009). Our results indicated that the induced tetraploid lines responded with significant higher antioxidant activities than their diploid progenitors, except for the cultivar 'Prompt'. This also agrees with the report from Meng et al. (2011), where increased antioxidant activities contributed to the tolerance of auto-induced tetraploid turnips over their diploid progenitors. Our results also showed a positive correlation between reduction in growth and the antioxidant activities suggesting that the tetraploid lines are better adapted in their first response to salinity stress.

Conclusions

1. Salinity stress significantly inhibits the germination of annual ryegrass (*Lolium multiflorum* spp. *multiflorum*) seeds. Chromosome duplication appears to have an important role during germination in saline conditions. The induced tetraploid lines in most cases had higher germination index and lower median germination time (T50) values than their diploid progenitors.

2. Polyploidy contributes to tolerance to salinity stress in annual ryegrass as observed in the morphological and physiological response at the vegetative stage. The induced tetraploid lines showed significant reduction in leaf growth and hence, were superior in their first response to salinity stress than their diploid progenitors.

3. The induced tetraploid lines of annual ryegrass had higher antioxidant activities than their parental diploids indicating that polyploidy contributes to tolerance to salinity stress that often leads to oxidative damage in plant cells.

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Atsako į druskingumo stresą skirtumai *Lolium multiflorum* diploidinių bei tetraploidinių augalų dygimo ir vegetatyvinio augimo tarpsniais

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Santrauka

Klimato kaita, gėlo vandens išteklių mažėjimas ir intensyvus drėkinimas yra vieni iš veiksnių, sukeliančių dirvožemių druskingumo didėjimą. Dėl druskėjimo procesų visame pasaulyje prarandami žemdirbystei tinkami plotai. Nors didžiausias dėmesys skiriamas maistinių augalų produkcijai, nukenčia ir pašarams skirti žolynai, kurie yra svarbi mitybos grandinės dalis.
Tyrimo tikslas – įvertinti gausiažiedės svidrės (*Lolium multiflorum* spp. *multiflorum*) diploidinių ir autotetraploidinių augalų morfologinį bei fiziologinį atsaką į druskingumo stresą dygimo ir augimo tarpsniais. Diploidinės ir indukuotos tetraploidinės sėklos buvo daigintos skirtingos koncentracijos druskos tirpaluose. Diploidinių ir tetraploidinių sėklų daigai 10 dienų kontroliuojamomis sąlygomis buvo veikiami 500 mM NaCl. Indukuotų tetraploidinių linijų augalų sėklos buvo didesnės ir pasižymėjo didesniais dygimo indekso bei vidutinio daigumo laiko (T50) įverčiais nei atitinkamos diploidinės tėvinės veislės. Vegetatyvinio augimo tarpsniu autotetraploidiniai augalai buvo atsparesni didesniam druskingumui, išsiskyrė didesniu santykiniu vandens kiekiu ir antioksidaciniu aktyvumu nei diploidinių veislių augalai.

Reikšminiai žodžiai: abiotinis stresas, antioksidacinis aktyvumas, gausiažiedė svidrė, ploidiškumas.

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Drought stress response of Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*) cultivars differing in their ploidy level

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Abstract

Drought is one of the critical abiotic stresses that significantly affect agricultural production and even more, current models predict an increase in its severity and intensity in the future. Generally, polyploidy has been found to improve the resistance of plants to abiotic stress. Understanding the role of ploidy in resistance to drought was achieved by comparing the response between diploids and their respective autotetraploids of Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*), hence maintaining the genetic homogeneity. Field trials were carried out in the 2017 and 2018 growing seasons and mild drought simulation experiments in controlled condition were also carried out to validate the effect of chromosome duplication. Results from morphological traits in the field experiment revealed that the induced tetraploids were taller, had longer inflorescence and larger flag leaf area than their diploid counterparts especially in the year 2018 characterized by prolonged drought. However, variations between diploids and induced tetraploid lines were observed in the resistance to short-period mild drought in the controlled experiment. Chlorophyll fluorescence (Fv:Fm), relative water content (RWC) and leaf wilting varied among the cytotypes, and also among cultivars. The induced tetraploid lines have significantly higher antiradical activity and phenolic content than the diploid progenitors indicating that increased ploidy level plays an important role in conferring resistance to drought in Westerwolths ryegrass.

Key words: mild-drought, morphological traits, phenolic contents, tetraploids.

Introduction

Many regions of the world are experiencing prolonged droughts that seriously threaten agricultural production. The frequency of various extreme climatological events are increasing due to climate change; all global climate models predict rising temperatures and consequently even higher risk of drought in many areas globally in the future either due to reduction in precipitation, an increase in evaporation, or a combination of these factors (Dai, 2013). A huge concern is placed on the impact of climate change on staple food production (Trnka et al., 2015), however, abiotic stresses affect forage production as well, pressing farmers and breeders to search for means of adapting the grasslands to arising challenges (Ergon et al., 2018).

Plants have developed different mechanisms to tolerate drought, such as dehydration avoidance and dehydration tolerance (Fang, Xiong, 2015). Dehydration avoidance occurs when plants slow down their growth rates, it involves various morphological and anatomical changes that reduce the loss of water through transpiration and also improve the uptake of water from the soil by modulation of root system architecture (Luo 2010). Dehydration tolerance is when plants are able to maintain their normal function under low leaf water status (Yates et al., 2019). Plants usually do this with accumulating both inorganic and organic substances to improve water retention by reducing the osmotic potentials (Blum, Tuberosa 2018).

At the molecular level, drought tolerance is a complex quantitative trait that involves the activation or suppression of many genes. Some of the genes are involved in antioxidation activity needed to produce substrates that reduce the elevated concentration of reactive oxygen species (ROS) arising due to drought (Zandalinas et al., 2018).

Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*), is a forage grass with high palatability and digestibility. It is fast growing due to its annual nature and produces high first cut yields. Besides the cultivation of Westerwolths ryegrass for the high-quality green feed or silage production, it can also be used as a catch crop (Humphreys et al., 2010). Westerwolths ryegrass, as well as its close relatives' perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*L. multiflorum* ssp. *italicum*), are self-incompatible and occur naturally as diploid (2 n = 2 x = 14), however autotetraploid induction is rather simple (Pašakinskienė 2000; Dabkevičienė et al., 2017). Tetraploid forage ryegrass vultivars are known to produce higher herbage yields (Humphreys et al., 2010; Burns et al., 2013; Kemesyte et al., 2017). Polyploid plants can exhibit higher adaptability, increased vigour and resistance to

unfavourable environmental factors compared to their diploid relatives (Sattler et al., 2016), yet some studies indicate that diploids are more resistant to abiotic stresses (Helgadóttir et al., 2018) or the differences are small (Kemesyte et al., 2017). Most research projects use plants with different pedigrees when exploring differences in yield, stress resistance and changes in transcriptome between diploids and tetraploids. This makes interpretation of the results more difficult – the changes in phenotypic traits or gene expression might be due to different genetic background instead of ploidy level. Comparing induced autotetraploids to their respective diploid parental lines helped to avoid this problem and gave more reliable insights into the effect of ploidy level on the performance of the plant.

The aim of the research is to compare the performance of diploid cultivars, induced autotetraploid lines and tetraploid cultivars of *Lolium multiflorum* ssp. *multiflorum* under natural and simulated drought conditions.

Materials and methods

Plant material. Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*) 8 diploid cultivars, 8 induced tetraploid lines and 5 tetraploid cultivars were used in our study (Table 1). Tetraploid induction was carried out as described in Akinroluyo et al. (2018).

Table 1. Westerwolths ryegrass cultivars and induced tetraploid lines used in the field trial (F) and drought stress trial in controlled environment (CE)

Name	Ploidy	Origin	Trial	Induced tetraploid name	Trial
Druva	diploid	LVA	F and CE	Druva-4x	F and CE
Varpė	diploid	LTU	F and CE	Varpė-4x	F and CE
Magloire	diploid	FRA	F and CE	Magloire-4x	F and CE
Top speed	diploid	FRA	F and CE	Top speed-4x	F and CE
Grazer	diploid	DEU	F and CE	Grazer-4x	F and CE
Surrey nova	diploid	USA	CE	Surrey nova-4x	CE
Prompt	diploid	FRA	CE	Prompt-4x	CE
Shoot	diploid	DNK	CE	Shoot-4x	CE
Wesley	tetraploid	DNK	F and CE	–	
Avance	tetraploid	DNK	F	–	
Rapid	tetraploid	RUS	F	–	
Caremo	tetraploid	DNK	F and CE	–	
Peleton	tetraploid	DNK	F and CE	–	

Field trial. The experiment was carried out in the fields of the Institute of Agriculture, Lithuania Research Centre for Agriculture and Forestry (55°40′ N, 23°87′ E) during the 2017 and 2018 growing seasons. The field experiment was established in three replicates in a randomized complete block design on May 25th in 2017 and May 9th in 2018, and each line / cultivar was represented by 20 plants per replicate. The soil of the experimental fields is *Endocalcari-Epihypogleyic Cambisol*, characterized by a homogeneous texture, pH_{KCl} 7.2, humus content 1.74%, available P_2O_5 175 mg kg^{-1} and K_2O 157 mg kg^{-1}. Fertilizers were applied before sowing $N_{30}P_{50}K_{70}$ and N45, and after cuts.

Plant height (cm), flag leaf area (cm^2) and inflorescence length (cm) were measured when the heading stage was completed (BBCH 59). Ten plants per replicate, three flag leaves and inflorescences per plant were measured using image processing program *ImageJ*. Infection with *Puccinia coronata* was visually scored. Fresh plant biomass was collected after regrowth from the first cut, dried and weighed to obtain the dry matter yield (DMY) from each cultivar and from the induced tetraploid lines.

The 2017 and 2018 growing seasons in Lithuania had different climatic conditions, especially in terms of the amount of rainfall and temperature, as shown in Figure 1. The summer of 2017 was rainy and cool, and the mean temperatures reached 20°C only at the end of July, whereas 2018 was much warmer, with less precipitation and lower air humidity; hence plants grown during 2018 were exposed to drought periods compared to the preceding growing season.

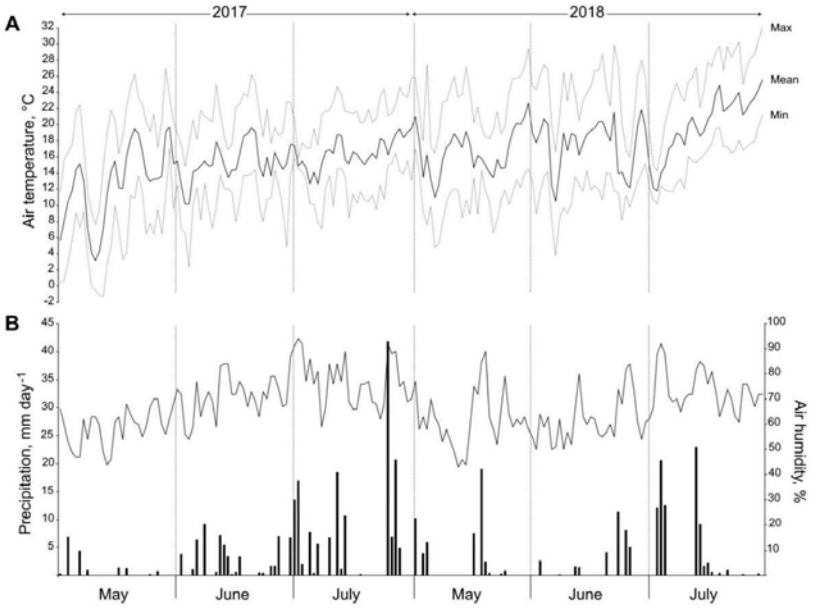


Figure 1. Meteorological conditions during 2017 and 2018: average, maximum and minimum air temperature, average precipitation and air humidity

Mild drought simulation in the controlled environment. Eight diploid cultivars, their respective induced tetraploid lines and three tetraploid cultivars were used in the drought simulation experiment (Table 1). The seeds were germinated on a filter paper and seedlings were allowed to grow for seven days before transplanting them to the round plastic pots (diameter 9 cm, height 8 cm) filled with 350g sandy soil (54% compost, 32% sand, 14% peat), five plants per pot, four pots per line/cultivar. The pots were placed at random in the phytotron, set to 24°C during the day and 18°C at night, a 16/8 hour photo-period and relative humidity of 60%. The plants were watered for two weeks and excess water was drained from holes at the base of the pots. Seven days before the inducing mild drought, 100 ml of water was added to each pot at the same time of the day to enable the plants to carry out their normal physiological functions. This was done to avoid excess water in the soil while preparing for drought initiation.

To investigate the effect of mild drought, 10 ml of water was added to each pot daily for five days. Five new unfolding leaves were marked at the nodes on the first day and the leaf elongation was recorded on a daily basis at exactly the same time. Leaf wilting was also observed at the end of the mild drought simulation and scored from no wilting to severely wilted: 1 – no sign of wilting, 6 – severely wilted. The experiment was carried out in three replicates. Severe drought commenced immediately after mild drought. Water was completely withheld for five days. The survival rate was determined.

Determination of the relative water content and chlorophyll fluorescence. The relative water content (RWC) was determined at the end of the drought treatment. Leaf samples were collected from each cultivar/line and weighed immediately to obtain the fresh weight (FW). The leaf samples were transferred to plastic sacks containing water and left for six hours before obtaining the turgid weight (TW). Finally, the leaf samples were blotted dry and placed in an oven at 70°C for 72 hours and weighed to obtain the dry weight (DW). The RWC was calculated using the Smart and Bingham (1974) formula:

RWC % = 100 [(FW – DW) / (TW – DW)].

The chlorophyll fluorescence (Fv/Fm) was measured using a chlorophyll fluorometer OS30p+ (Opti-Sciences Inc. USA).

Antiradical activity measurement. 2,2-diphenyl-1-picrylhydrazyl (DDPH) free radical scavenging activity was determined by modifying the method described in Brand-Williams et al. (1995). The leaf samples were collected after mild drought and dried in an oven at 40°C for four days, homogenised and 0.5 g of the homogenised leaves was suspended in 70% methanol. The extraction was done in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH and Co. KG, Germany) for 60 min at 50°C and 480 w. 2 ml of DDPH solution in 70% v/v methanol was mixed in 2 μL methanol extract. The reduction in absorbance at 515 nm was measured and expressed as Trolox equivalent antioxidant capacity.

Determination of total phenolic content. Spectrophotometric measurements were carried out with a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, USA). The total phenolic content (mg GAE/100 g DW) in the methanol (99.0%, v/v) of Westerwolths ryegrass leaves was determined by the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE).

Statistical analysis. Pairwise t-test, analysis of variance (ANOVA) with post hoc Duncan multiple range tests were calculated with using software *SAS* (Statistical Analysis System). Pearson's correlation coefficients were used to investigate relationships between selected variables. Mean ± SE (standard error of mean) were used to describe the variability of measurements.

Results

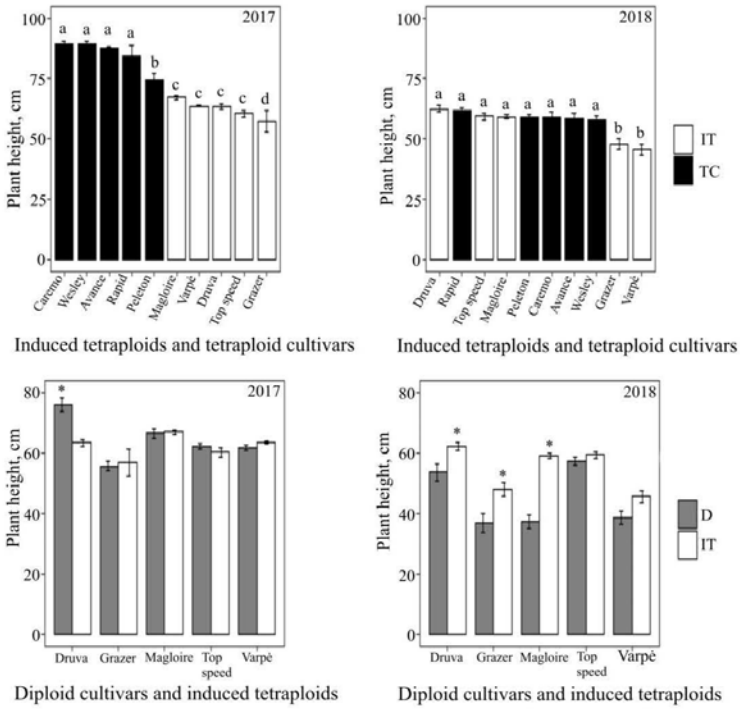
Analysis of variance. The year, ploidy level and cultivars were taken as the main factors. The effect of ploidy and cultivars were not significant for the morphological traits but the year effect was highly significant ($p \leq 0.01$). This indicated that the different weather condition in 2017 and 2018 had the largest effect on the morphological traits. To determine the effect of ploidy on the morphological traits, the model cultivar + ploidy + replicate + cultivar × ploidy + error was applied for each year separately. The ploidy level and cultivar were considered as fixed factors. Result of the ploidy, cultivar and their interaction effect analysis are presented in Table 2.

Table 2. The effect of ploidy and their interaction effects on the Westerwolths ryegrass traits between diploids and induced tetraploids as indicated by the *F* values

Trait	Year	C (df = 4)	P (df = 1)	R (df = 2)	P × C (df = 4)
Plant height	2017	12.94**	1.30	5.64**	5.64**
	2018	17.96**	12.82**	3.20	40.43**
Flag leaf area	2017	12.62**	1.64	2.26	41.46**
	2018	4.23**	102.66**	0.63	74.15**
Inflorescence length	2017	37.14**	14.74**	0.95	58.04**
	2018	68.72**	168.14**	0.67	177.52**

df – degree of freedom, C – cultivar, P – ploidy level; ** – indicate significant difference and $p \leq 0.01$

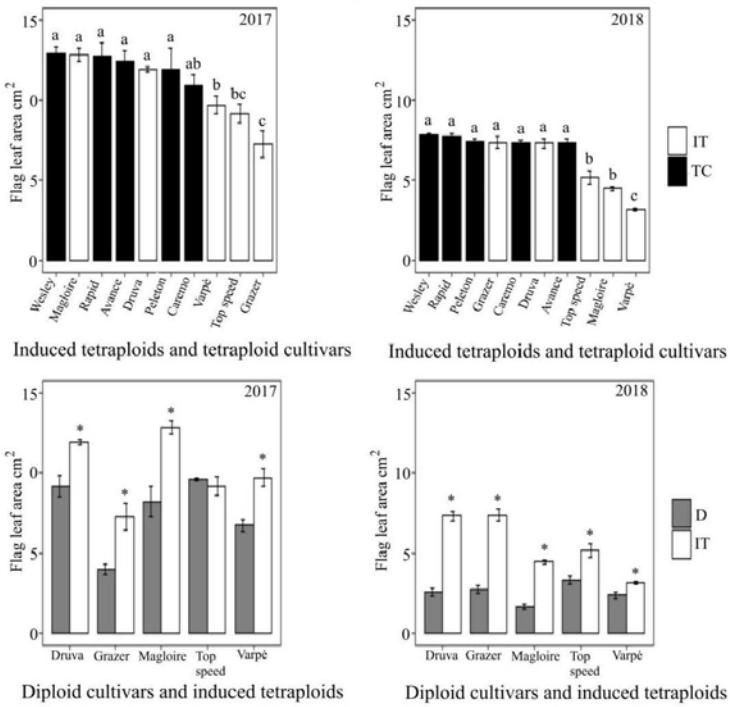
Comparison of the morphological traits between the diploid cultivars, induced tetraploids and tetraploid cultivars in the field trials. Plant height. No significant difference ($p > 0.05$) was observed in the plant height between the diploids and their corresponding induced tetraploid lines in 2017 field trials except in the cultivar 'Druva' which the diploids were significantly higher ($p \leq 0.05$) than the induced tetraploid counterpart (Fig. 2). The tetraploid cultivars performed better and were significantly higher than both the diploid cultivars and induced tetraploids. However, the 2018 field trials that had lower amount of rainfall and increased temperature, significant differences ($p \leq 0.05$) were observed in the plant height of diploids and induced tetraploids with most of the induced tetraploids performing better and showing higher plant vigour than their diploid progenitors.



Note. Data shown as mean ± standard error of three replicates; * – represents significant difference at $p \leq 0.05$ pairwise *t*-test between diploids and corresponding induced tetraploids; different letters indicate significant differences at $p \leq 0.05$; Duncan's multiple range tests.

Figure 2. The plant height of diploid cultivars (D) induced tetraploid lines (IT) and tetraploid cultivars (TC)

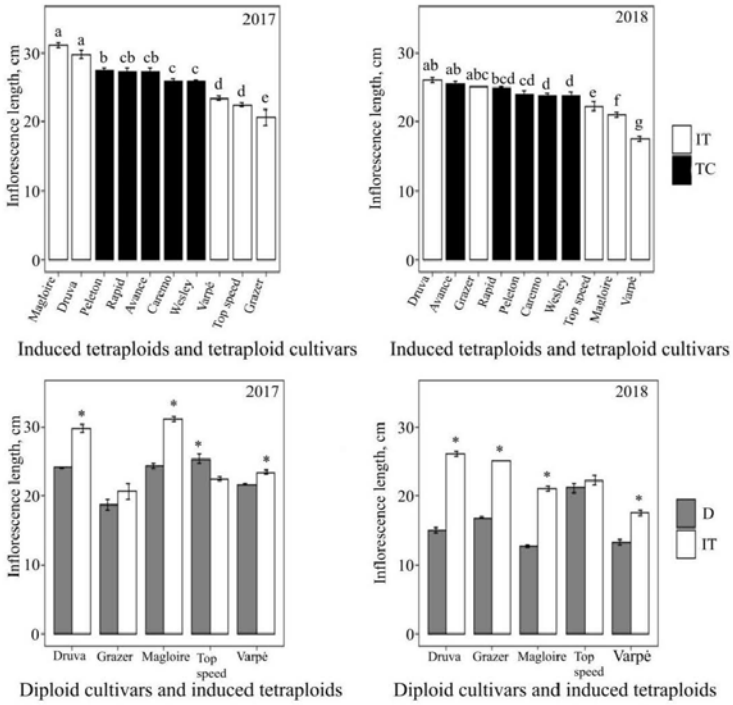
Flag leaf area. We evaluated the variations in the area of the flag leaf in diploid cultivars, their respective induced tetraploid lines and tetraploid cultivars. The induced tetraploid lines had flag leaves with larger surface areas than the diploid cultivars in both growing seasons. However, drought periods in 2018 apparently affected the flag leaf area of both cytotypes (Fig. 3). The tetraploid cultivars had flag leaves with larger surface areas than most of the induced tetraploid lines.



Note. Data shown as mean ± standard error of three replicates; * – represents significant difference at $p \leq 0.05$ pairwise *t*-test between diploids and corresponding induced tetraploids; different letters indicate significant differences at $p \leq 0.05$; Duncan's multiple range tests.

Figure 3. The flag leaf area of diploid cultivars (D) induced tetraploid lines (IT) and tetraploid cultivars (TC)

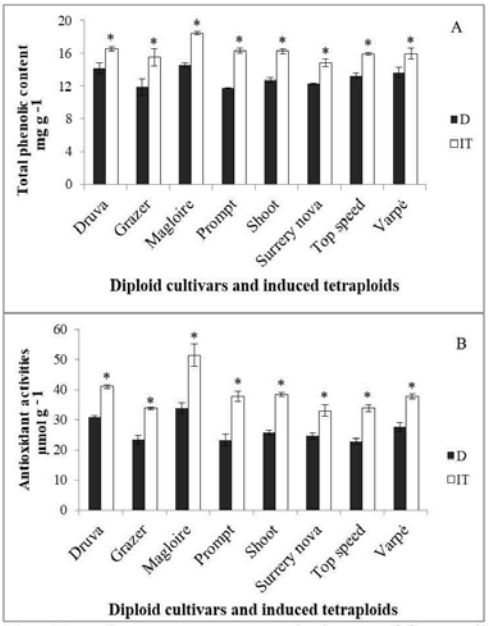
Inflorescence length. The induced tetraploids also had a longer inflorescence length in both years when compared to their parental diploids except in the cultivar 'Top speed' as shown in Figure 4. Drought significantly affected the inflorescence length of both cytotypes in the second year of investigation. However, most of the tetraploid cultivars have longer inflorescence than the induced tetraploid lines in both growing seasons.



Note. Data shown as mean ± standard error of three replicates; * – represents significant difference at $p \leq 0.05$ pairwise *t*-test between diploids and corresponding induced tetraploids; different letters indicate significant differences at $p \leq 0.05$; Duncan's multiple range tests.

Figure 4. The inflorescence length of diploid cultivars (D) induced tetraploid lines (IT) and tetraploid cultivars (TC)

Physiological and morphological response to mild drought. The phenolic content and antiradical activity in response to mild drought were determined in both diploids and the induced tetraploids. The result showed that the induced tetraploid lines significantly produced more phenolic compounds and also had more antiradical activity response to mild drought ($p \leq 0.05$) than their diploid progenitors as shown in Figure 5.



Note. Data shown as mean ± standard error of four replicates; * –significant differences between diploid cultivars and respective induced tetraploid lines $p \leq 0.05$ pairwise t-test.

Figure 5. The total phenolic content (A) and antiradical activity (B) of diploids and induced tetraploid lines after 5 days mild drought

The leaf elongation was measured daily, growth reduction, relative water content, the chlorophyll fluorescence and the wilting score were determined at the end of the mild drought simulation experiment and the data is presented in Table 3.

Table 3. Comparison of leaf length, leaf relative water content chlorophyll fluorescence (Fv:Fm) and wilting score of diploid cultivars induced tetraploid lines and tetraploid cultivars after the mild drought simulation

Line / cultivar	Leaf length cm	Leaf length control cm	Leaf length reduction %	Relative water content %	Fv:Fm	Wilting score
Magloire 2x	8.8 ± 0.15	13 ± 0.24	32 ± 0.00	71.8 ± 0.82	0.51 ± 0.03	5
Magloire-4x	9.8 ± 0.31*	15 ± 0.29*	35 ± 0.02	79.6 ± 0.92*	0.63 ± 0.00*	2
Surrey-nova 2x	9.0 ± 1.22	14.3 ± 0.29	37 ± 0.08	79.9 ± 0.38	0.59 ± 0.03	3
Surrey-nova-4x	8.8 ± 0.70	14.4 ± 0.37	39 ± 0.04	80.8 ± 0.81	0.65 ± 0.01	5
Varpė 2x	8.8 ± 1.10	12.3 ± 0.49	29 ± 0.07	80.5 ± 0.30	0.61 ± 0.03	4
Varpė-4x	7.4 ± 0.90	14.2 ± 0.28*	48 ± 0.05*	79.5 ± 0.48	0.62 ± 0.03	3
Grazer 2x	7.8 ± 0.24	11.2 ± 0.32	31 ± 0.04	79.5 ± 0.47*	0.65 ± 0.00	3
Grazer-4x	8.8 ± 0.19*	14.7 ± 0.37*	40 ± 0.04*	73.6 ± 1.11	0.62 ± 0.01	6
Shoot 2x	11.3 ± 0.11*	13.2 ± 0.17	15 ± 0.01	79.1 ± 0.44	0.51 ± 0.01	3
Shoot-4x	9.9 ± 0.22	13.4 ± 0.11	26 ± 0.01*	84.3 ± 0.00*	0.60 ± 0.02*	2
Prompt 2x	8.9 ± 0.32	12.6 ± 0.16	29 ± 0.02	78.6 ± 0.84*	0.66 ± 0.01*	3
Prompt-4x	10.2 ± 0.45	13.8 ± 0.32*	26 ± 0.02	73.3 ± 0.96	0.51 ± 0.01	6
Top speed 2x	8.3± 0.21	11.7 ± 0.22	31 ± 0.00	80.5 ± 0.96	0.65 ± 0.01*	4
Top speed-4x	8.1± 0.20	13.7 ± 0.28*	42 ± 0.00*	78.1 ± 0.96	0.53 ± 0.02	3
Druva 2x	9.2± 0.18	11.7 ± 0.35	23 ± 0.01	75.7 ± 0.84	0.64 ± 0.02	3
Druva-4x	7.6± 0.62	13.4 ± 0.28*	44 ± 0.03*	77.2 ± 0.93	0.64 ± 0.01	5
Caremo	10.0± 0.17	14.8 ± 0.19	32 ± 0.02	82.9 ± 0.00	0.59 ± 0.05	3
Wesley	10.2± 0.10	13.6 ± 0.22	25 ± 0.02	81.9 ± 1.28	0.58 ± 0.00	3
Peleton	11.0± 0.24	14.4 ± 0.43	24 ± 0.01	81.0 ± 0.96	0.67 ± 0.01	2

Note. Data shown as mean ± standard error of three replicates; the means followed by * between diploids and corresponding induced tetraploid lines are significantly different at lines $p \leq 0.05$ pairwise *t*-test.

We studied in detail the leaf elongation of plants grown under control and stress conditions and evaluated the percentage decrease in leaf elongation. 5 induced tetraploid lines had a significant reduction in leaf elongation than their diploid progenitors ($p \leq 0.05$). This reduction in leaf elongation correlates with the total phenolic content in the leaves ($r = 0.45$, $p \leq 0.01$) as shown in Table 4. Variations in the relative water content and chlorophyll were observed between cytotypes.

Table 4. Correlation coefficients matrix among the physiological traits in response to mild drought

	Antiradical activity	Phenolic content	Fv:Fm	Relative water content	Leaf length reduction
Leaf length reduction	0.28**	0.45**	−0.09**	−0.19**	1
Relative water content	0.14**	0.17**	0.17**	1	−0.19**
Fv:Fm	0	−0.06**	1	0.17**	−0.09**
Phenolic content	0.75**	1	−0.06**	0.17**	0.45**
Antiradical activity	1	0.75**	0	0.14**	0.28**

** – correlation is significant at $p \leq 0.01$

A significant medium positive correlation was found between morphological traits measure in 2018 field trials the phenolic content during mild drought ($p \leq 0.01$). Similar relationship was also found between the antiradical activity and morphological traits as shown in Table 5.

Table 5. Correlation coefficients matrix between physiological response during mild drought and morphological traits in the 2018 growing season

	Plant height	Flag leaf area	Inflorescence length
Antiradical activity	0.49**	0.46**	0.41**
Phenolic content	0.53**	0.53**	0.51**

** – correlation is significant at $p \leq 0.01$

Discussion

The prominence of polyploids in grass species is an indicator that polyploid has some adaptive importance. Polyploidy has been widely studied in many plant species and is known to often demonstrate phenotypes that are not present in the diploid progenitors (Ramsey, Schemske, 2002). These polyploidy induced traits, such as drought tolerance and increased biomass production, could be advantageous in many agricultural processes. Although the mechanism by which the novel traits are manifested in polyploid plants is still not properly understood, neofunctionalisation of genes has been a long standing theory according to which genes acquire a new function after gene duplication (Osborn et al., 2003).

In both years of investigation, the diploid and tetraploid cultivars demonstrated variations in their growth patterns. In 2017 field trials, the tetraploid cultivars were higher than both the induced tetraploid lines and the diploid cultivars. The functional divergence resulting from polyploidisation often confers selective advantages to polyploids (Osborn et al., 2003). These advantages are rarely immediate but occur over a period of time, and this could be a reason why no significant difference was observed in the plant height of induced tetraploid lines and diploid progenitors. Other studies, however, have reported that the effect of polyploidisation is immediate. The tetraploid cultivars may have performed better than the induced tetraploid lines, especially in terms of plant height, as a result of genomic stability and epigenetic changes such as DNA methylation and histone modification, which can be inherited in gene expression (Liu, Wendel, 2003). Dar et al. (2013) reported that the DNA methylation changes increased from first generation of induced tetraploids to the fourth generation in Phlox drummondii. DNA methylation has also been found to play a role in genome stabilisation and the expression of redundant genes (Adams et al., 2003). DNA methylation also plays an essential role in plant development (Aversano et al., 2013). In essence, the induced tetraploids could perform better in subsequent generations when the DNA methylation increases and the genome stabilises.

The leaf growth is a dynamic process often involving independent pathways that direct the cell components (Gao et al., 2016). Previous studies have shown that autotetraploid of *Lolium* species had long leaves due to the increase in cell length and a faster rate of cell elongation (Sugiyama, 2005). The increase in the flag leaf area of the induced tetraploid lines compared to the diploid progenitors can likely be attributed to an increase in the size of cells as a result of genome duplication and a faster rate of elongation. Also, it could be suggested that DNA methylation resulting from chromosome duplication could lead to a differential gene expression level resulting in the observed changes in the plant morphology of tetraploids. In the present study, the effect of drought on the flag leaf was clearly visible in both cytotypes. The flag leaves in 2018 field trials had a smaller surface area in both cytotypes when compared to the result obtained in the previous year. This reduction in the area of the flag leaf is an adaptive response to drought. However, the reduction in the flag leaf area was more profound in the diploid cultivars. The

induced tetraploid lines also had a longer inflorescence length in both years when compared to their parental diploid except in the cultivar 'Top speed'.

Renny-Byfield and Wendel (2014) suggested that immediate and long-term disturbances in the genome, transcriptome, and epigenome are intrinsic to polyploidy. This may explain why the induced tetraploid lines were not taller than their respective diploid progenitors in the 2017 growing season and probably why the tetraploid cultivars had a better performance compared to the induced tetraploid lines in 2017. The genetic makeup of the cultivars/lines appears to contribute to the variations observed in the field performance of the cultivars/induced tetraploid lines.

Drought generally increases the production of reactive oxygen species (ROS) in plants, and if cells are poorly protected this ROS could damage membrane lipids, protein and also DNA molecules, leading to cell death. Redox homeostasis occurs when there is equilibrium in the production and scavenging of ROS. Aghaei et al. (2009) suggested that the increase in stress tolerance correlates to increased antiradical activity. Our results showed that the induced tetraploids had significantly more antiradical activity than their diploid progenitors. These results are similar to the reports from Meng *et al.* (2011), where an increase in antiradical activity resulted in increased stress tolerance in auto-induced tetraploid of turnips compared to their diploid counterparts.

Phenolics are secondary metabolites that influence different physiological processes related to growth and development (Tanase et al., 2019). The production of secondary metabolites is often triggered by drought stress, and studies have reported that increases in the production of phenolic compounds such as quercetin and rutin have contributed to tolerance to drought stress in *H. brasiliense* (Abreu, Mazzafera, 2005). Our results suggested that the induced tetraploids produced significantly more phenolic compounds and had more antiradical activities compared to their parental diploids during mild drought. These could play an important role in long term exposure to drought, as the induced tetraploid lines could be at an advantage over their diploid progenitors.

Plant tolerance to drought occurs via many mechanisms such as dehydration avoidance and dehydration tolerance (Fang, Xiong, 2015). Results from mild drought simulation showed that the cultivars exhibited signs of dehydration avoidance as a first response to drought by reducing their leaf growth. 5 induced tetraploid lines had a significant reduction in leaf growth compared to their diploid counterpart. This response to drought stress involves a cascade of reactions involving many genes at the molecular level; however, duplication of the genetic materials seems to have an advantage over the diploid cultivars in the first response to drought in Westerwolths ryegrass.

Plants respond to water stress by complex mechanism inducing various physiological, morphological, biochemical and molecular changes. These responses are highly varied among plant species and also between cytotypes (Aslam et al., 2015). Leaf chlorophyll fluorescence reflects the integrity of photosynthetic apparatus or photochemical efficiency of the photosystem II system in the light reaction of photosynthesis (Kaiser, 1987). Studies have used chlorophyll fluorescence and leaf water content to evaluate the drought response different grass species (Merewitz et al., 2011; Shukla et al., 2015). In this study, variations were found in chlorophyll fluorescence across cultivars. Variations were also observed in the relative water content of the diploid and their corresponding induced tetraploid lines, however, no significant correlation was found between the traits observed during mild drought.

We correlated the morphological and physiological parameters (reduction in leaf elongation, relative water content, chlorophyll fluorescence, antiradical activity and phenolic content) at the end of mild drought simulation and found no strong correlation among these parameters except a medium positive correlation between the growth reduction and phenolic contents and strong positive correlation between the antiradical activity and phenolic content ($r = 0.75$, $p \leq 0.01$). Large group of phenolic compounds have antioxidant properties (Rani et al., 2018) and this could explain why the phenolic contents significantly correlated positively with antiradical activity. We also carried out a correlation between the antiradical activity and phenolic contents at the end of mild drought with the morphological traits in 2018 field trials characterized by a lower amount of rainfall. All the morphological traits had a significant medium positive correlation with the antiradical activity and phenolic contents ($p \leq 0.01$).

Drought is a complex quantitative trait and previous studies have shown that drought tolerance could vary at different developmental stages (Kron et al., 2008). In addition, the phenotypic response of plants to stress are complex and governed by the interactive effects of factors such as stress duration, genotypes, developmental stage at which the stress occurs and the intensity of the stress (Obidiegwu et al., 2015). Yet, Boutraa et al. (2010) reported that genotypes exhibiting high DPPH scavenging activity and high phenolic content at the seed stage continued to show high antiradical activity and phenolic contents at other stages when working on drought tolerance in wheat cultivars. This is a drought tolerance mechanism. In this study, the induced tetraploid lines had more antioxidant and phenolic contents in response to mild drought. This difference in physiological response at the seedling stages could proceed to other developmental stages and contribute to the observed improved performance of the induced tetraploid lines in the 2018 field trials. However, future work will be done on the DPPH scavenging and total phenolic contents in field trials at various developmental stages.

Wilting is a physiological and morphological response to drought stress that occurs due to the loss of cell turgor pressure, resulting in the drooping of leaves (Tavakol, Pakniyat, 2007). Wilting under drought stress is common phenomenon and this visual cue is important for assessing drought tolerant plants. As a result of the increased reduction in leaf elongation in the induced tetraploids during mild drought, it was assumed that the induced tetraploid plants will have a lower wilting score than their diploid progenitors, however, this was true in just one induced tetraploid (Shoot-4x), which had a lower wilting score than the diploid progenitors and a higher

reduction in leaf growth. Three induced tetraploids (Grazer-4x, Top speed-4x and Druva-4x) had a higher wilting score despite their high reduction of leaf growth when compared with their diploid progenitors. There was no significant difference between Magloire and Magloire-4x in the leaf growth reduction, but the induced tetraploid had a higher relative water content, Fv/Fm and a lower wilting score. This suggests that the Magloire-4x was able to tolerate drought better than their diploid progenitors. This superior trait demonstrated by Magloire-4x was also apparent in the field experiment and could be associated with many factors, including dosage effect, the neo-functionalisation of duplicated genes, increased allelic diversity and mutation buffering (te Beest et al., 2012).

Comparing results from cultivars/induced tetraploid lines in our field experiment in 2018 and the drought simulation experiment, it is not surprising that the tetraploid cultivars and induced tetraploid lines with a lower wilting score showed a better phenotypic character in the field experiments than the diploid cultivars. However, the induced line 'Grazer-4x' and 'Druva-4x' that both had a high wilting score still had a better phenotypic character than their diploid progenitors. In field experiments, the process of dehydration usually progresses slowly and induces the minimal drought-related genes. Conversely, the rapid stress imposition of drought in simulation pots with restricted roots often this triggers greater gene expression (Barker et al. 2005). In addition to the genotypic difference between cultivars, one possible explanation for the observed high wilting score for 'Grazer-4x' and 'Druva-4x' could be related to plant size. Large plants wilt faster than smaller plants when irrigation is stopped in pot experiments (Blum, 2011) although this is not consistent with other induced tetraploid lines.

Homeostasis can be described as the ability of organisms to adjust their internal physiological condition when responding to a changing external environment (Wang et al., 2016). In this regards, our empirical data on the phenotypic traits suggested that, the tetraploid cultivars and induced tetraploid lines could be at a homeostatic advantage over their diploids counterparts. This implies that ploidy influences physiological response that improves drought tolerance in Westerwolths ryegrass.

With the abundant supply of both diploid and tetraploid forage grass cultivars on the market, making it rather tricky to take an informed decision for the farmer. The breeders, on the other hand, are faced with the never-ending challenge to steer the breeding programs to meet the challenges that might arise in the future. Current climatic trends and future models indicate an increase of the areas facing constant or recurrent droughts making breeding for drought-tolerant forage cultivars a top priority.

Conclusions

1. The increase in ploidy confer advantages in the performance of the induced tetraploid lines in the field trials as observed in the morphological characteristics especially in water deficit conditions when compared to diploid progenitors.

2. The induced tetraploid lines of Westerwolths ryegrass had higher antiradical activity and total phenolic content during mild drought periods than their parental diploids indicating that polyploidy contributes to tolerance to drought that often leads to oxidative injury in plant cells.

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Skirtingo ploidiškumo gausiažiedės vienametės svidrės (*Lolium multiflorum* spp. *multiflorum*) genotipų atsakas į sausros stresą

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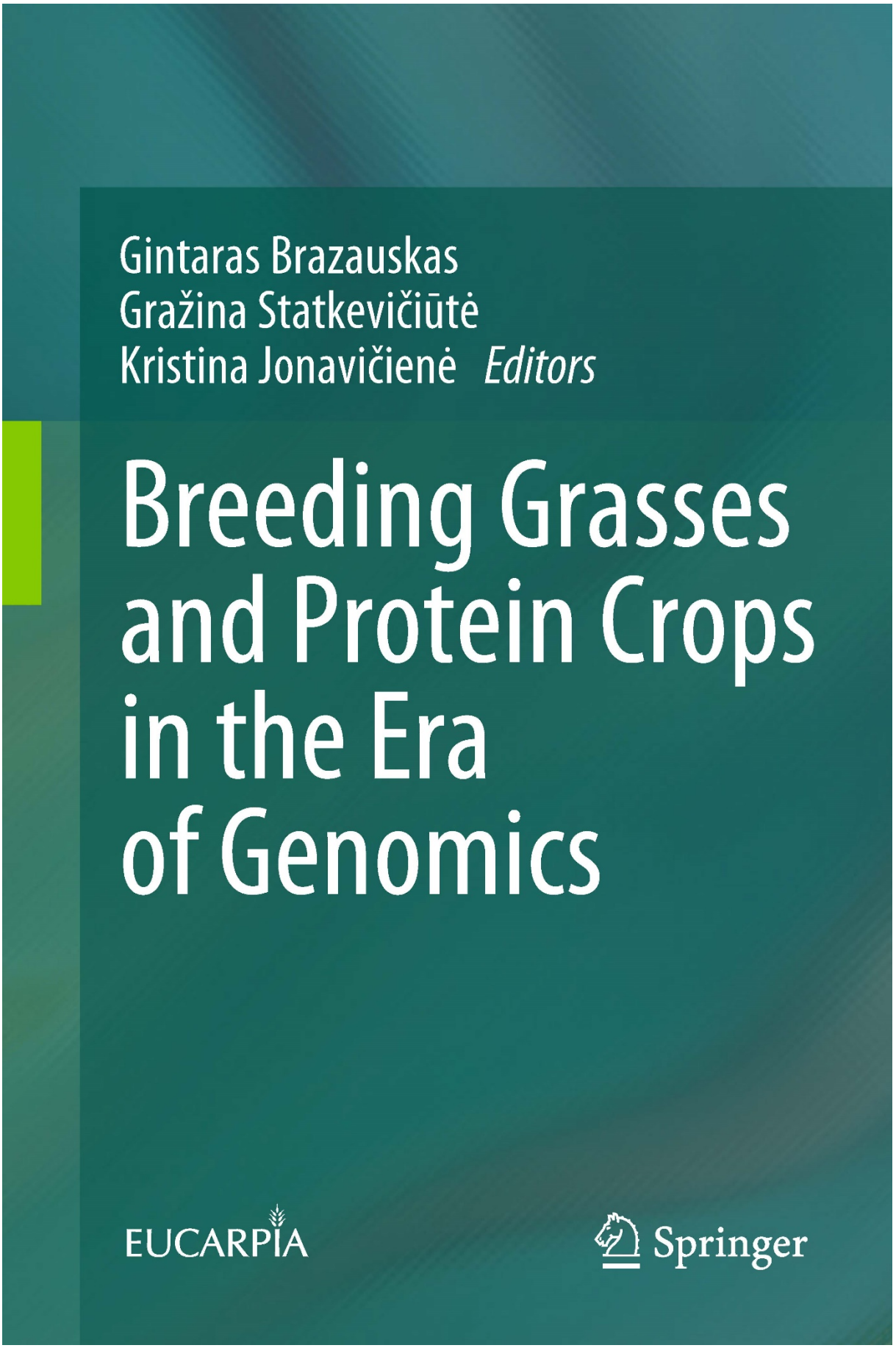
Santrauka

Sausros stresas yra vienas svarbiausių abiotinių veiksnių, sukeliančių reikšmingus žemės ūkio produkcijos nuostolius. Remiantis klimato kaitos modeliais, ateityje ši problema tik aštrės. Poliploidiniai augalai dažnai yra atsparesni abiotiniams stresams. Siekiant įvertinti ploidiškumo įtaką augalų atsparumui, tyrimo metu buvo palygintas diploidinių gausiažiedės vienametės svidrės veislių ir autotetraploidinių linijų atsakas į sausros stresą. Autotetraploidinės linijos buvo indukuotos iš diploidinių veislių, siekiant užtikrinti kuo didesnį genetinį skirtingo ploidiškumo augalų vienodumą. Chromomosomų duplikacijos poveikis augalo morfologiniams požymiams ir sausros atsakui buvo vertinami 2017 ir 2018 m. natūraliomis sąlygomis lauko eksperimente ir kontroliuojamos aplinkos sąlygomis fitotrone, simuliuojant trumpalaikę sausrą. Lauko eksperimente tetraploidiniai augalai buvo aukštesni, formavo ilgesnius žiedynus ir didesnį sausųjų medžiagų derlių, palyginus su diploidinėmis veislėmis. Šie skirtumai tarp ploidiškumo grupių ypač išryškėjo sausringais 2018 m. Kontroliuojamomis sąlygomis nustatyta ženkli chlorofilo fluorescencijos (Fv:Fm), santykinio vandens kiekio lapuose ir lapų vytimo įverčių variacija tarp genotipų ir ploidiškumo grupių. Reikšmingai didesnis antioksidacinis aktyvumas ir fenolinių junginių kiekis indukuotuose tetraploiduose, palyginus su diploidais, patvirtino poliploidiškumo įtaką atsparumui sausrai gausiažiedės vienametės svidrės augaluose.

Reikšminiai žodžiai: morfologiniai požymiai, tetraploidai, trumpalaikė sausra.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Tetraploid Induction in *Lolium multiflorum*

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Abstract. The induction of tetraploids from diploid cultivars of *L. multiflorum* is important in improving the germplasm. The aim of the study is to investigate the optimal concentration and time duration to induce tetraploids from diploid cultivars of *L. multiflorum*. Seedlings developing from excised embryos were subjected to different concentration of mitosis inhibitors and for different time intervals. The survival and induction percentages were determined for each induction approach.

Keywords: Annual ryegrass · Colchicine · Polyploidization

1 Introduction

Annual ryegrass (*Lolium multiflorum ssp. multiflorum*), also called Westerwolths ryegrass, is a leafy highly tillering grass of high palatability and digestibility. Annual ryegrass is also known to be one of the reliable cool-season grasses that have leaves that are rich in protein, vitamins and minerals in addition to being highly digestible and palatable to grazing animals (Humphreys et al. 2010).

Annual ryegrass occur naturally as diploids ($2n = 2x = 14$). However, the production of polypoid plants has been of interest to grass breeders to obtain differentiated genotypes to improve and maximize agronomic traits. Plants with different ploidy levels may differ in their growth habit, morphologically, physiological, cellular and biochemical aspects (Leitch and Leitch 2008).

Polyploid plants have been found to show a better resistance to both biotic and abiotic stress. In addition, polyploid plants have been found to display superior agronomic traits when compared to their diploids counterparts such as having a better tolerance to environmental stress (Comai 2005). These properties of polyploids enable them to be better adapted to a wider ecological range (Blanc and Wolfe 2004).

2 Materials and Methods

This study was performed at the Institute of Agriculture, Lithuanian Research Center for Agriculture and Forestry, Laboratory of Genetics and Physiology. Ten different cultivars of *L. multiflorum spp. multiflorum* were used (Table 1).

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Table 1. List of cultivars used for polyploid induction.

Cultivar	Ploidy	Origin
Druva	2n	Latvia
Varpe	2n	Lithuania
Magloire	2n	France
Prompt	2n	France
Aramo	2n	France
Top speed	2n	France
Surrey nova	2n	USA
Weldra	2n	Netherlands
Grazer	2n	Germany
Shoot	2n	Denmark

The method described by Pašakinskienė (2000) was adopted for the sterilization of seeds and tetraploid induction. Seeds were surfaced sterilized and the embryos were excised from the seeds. Sterilized embryos were sprouted in a petri dish containing Gamborg B5 (Duchefa Biochemie) medium for 3–5days at a temperature of 24 °C. The coleoptiles were allowed to grow till up to 0.5 cm long and then transferred to a 4 °C refrigerator for 2 day to pause the process of mitosis. Prior to the treatment of plants with colchicine and amiprophos methyl (APM), the plants were transferred to growth chamber at 28 ° C for 1 h. The concentration and duration of the inhibitors are shown in Table 2.

Table 2. Treatment used for tetraploid induction in *Lolium multiflorum spp. multiflorum*

Mitosis inhibitors	Concentration	Duration
Colchicine	10 mM	3 or 4 h
Colchicine	8 mM	3 or 4 h
Amiprophos methyl	0.1 mM	4 h
Amiprophos methyl	0.05 mM	4 h
Amiprophos methyl	0.04 mM	4 h
Amiprophos methyl	0.03 mM	3 h
Amiprophos methyl	0.02 mM	3 h
Amiprophos methyl	0.015 mM	3 h

The ploidy levels of the survived plants were checked using a Partec PA flow cytometer. Also, a root tip squash technique for counting the chromosome number were used to verify the ploidy levels of 15 randomly selected plants

3 Results and Discussion

Chromosome doubling was achieved in a varying degree using different mitosis inhibitors however, the survival and induction rate depends on the affinity and toxicity of the inhibitors. In our experiments, different tetraploid induction rates were obtained using

different concentrations and exposure times. Results from the Druva and Grazer cultivars of *L. multiflorum spp multiflorum* (Table 3) showed that both colchicine and APM were capable of inducing tetraploids from diploid cultivars. However, APM in higher concentrations appeared to be highly toxic to the plants. APM concentration of 0.05 mM with a 4 h exposure resulted in 12% survival rate of the treated plants but reducing the exposure time to 3 h and the concentration to 0.15 mM resulted in a 38.5% survival rate with a 40% induction rate in the Druva cultivar.

Table 3. Comparison of the survival and induction of tetraploid rate in 2 genotypes using amiprophos methyl and colchicine at different concentrations and exposure time

Mitosis inhibitors in various conc. time interval and temp.	Survival rate%		Induction rate%	
	Druva	Grazer	Druva	Grazer
10 mM colchicine, 4 h	24.5	28.8	71.4	61.4
8 mM colchicine, 3 h	59.7	52.5	25.6	37.1
10 mM colchicine, 3 h	40.7	63.3	68.1	61.4
8 mM colchicine, 4 h	41.2	32.9	42.5	55.8
0.1 mM amiprophos methyl, 4 h	0	0	0	0
0.05 mM amiprophos methyl, 4 h	12.5	14.9	57.1	50.0
0.04 mM amiprophos methyl, 4 h	21.7	24.0	30.8	45.8
0.03 mM amiprophos methyl, 3 h	17.0	18.9	37.5	44.4
0.02 mM amiprophos methyl, 3 h	22.2	23.2	30.0	31.8
0.015 mM amiprophos methyl, 3 h	38.5	–	40	–

The survival rate with colchicine and maximum tetraploid induction rates appeared to be better than with amiprophos methyl. A similar experiment to compare and evaluate the efficiency of colchicine and amiprophos methyl on double haploid production of onions was done by Foschi et al. (2013). Their findings revealed that colchicine was more efficient in doubling chromosomes than amiprophos methyl at the same exposure time although a higher concentration of colchicine was necessary to induce polyploidy than amiprophos methyl.

Weiler et al. (2014), found that treating seeds with mitosis inhibitors was more effective that seedling treatment of *Paspalum notatum*. They observed that treating seedlings with colchicine concentration of 0.1% and higher and for a longer duration (18–24 h) was highly toxic to the plants. Also, Pereira et al. (2014) showed that tetraploid induction in *Lolium multiflorum* using 15–20 day old seedlings was not possible at a concentration of 12.5 mM colchicine for 24 h. The optimal concentration resulted in 32% survival rate and 27% induction rate when the treatment is composed of 1% of DMSO in solution with 12.5 mM colchicine for 24 h (Pereira et al. 2014). However, our experiment showed that a high rate of tetraploid induction with lower concentrations of colchicine and a shorter exposure time was achievable. The optimal concentration for tetraploid induction in *Lolium multiflorum* (Grazer) is achieved using colchicine treatment with a concentration of 10 mM for 3 h which resulted in a 63.3% survival rate and a 61.4% induction rate.

Many factors such as the concentration of colchicine, plant genotype, the exposure time and the treated seedling organ have been to found to determine the efficiency of colchicine in inducing polyploids in *Rosa* species (Khosravi et al. 2008). Based on the results reported in Table 3, two combinations of colchicine concentration and exposure time were chosen to induce tetraploids in 8 different genotypes of *L. multiflorum*. Significant differences in the survival and induction rate among cultivars were observed.

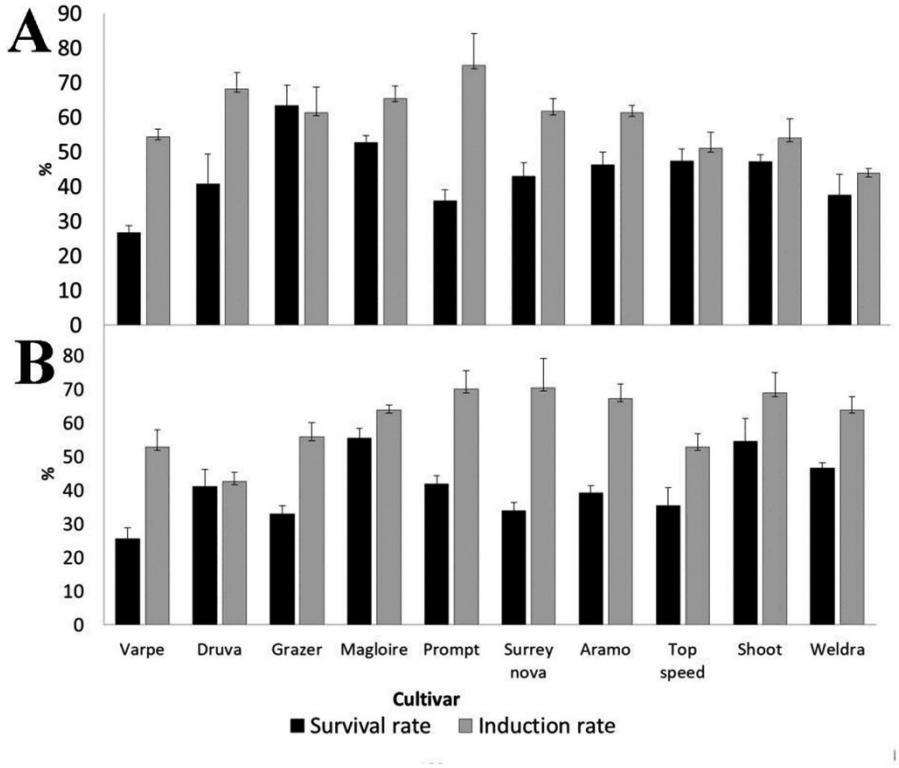

Fig. 1. Colchicine efficiency in inducing tetraploids in 10 diploid cultivars at different concentration and exposure time A (0.01 M colchicine, 3 h) B (0.008 M colchicine, 4 h). The error bar represents the standard error.

4 Conclusion

Induction of tetraploids from diploid cultivars of *L. multiflorum* can be achieved using both colchicine and APM. However, the efficiency of the mitosis inhibitors depends on the optimal concentration, exposure time, affinity and toxicity of the inhibitors. A concentration of 0.01 M and 0.008 M with an exposure time of 3 and 4 h respectively was found to be most efficient in inducing tetraploids from diploid cultivars of *L. multiflorum*.

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SANTRAUKA

ĮVADAS

Abiotiniai stresai neigiamai veikia žemės ūkio produktyvumą visame pasaulyje, ženklūs nuostoliai patiriami dėl ilgėjančių sausringų periodų. Prognozuojama, kad žmonių populiacija 2050 metais gali pasiekti 9 milijardus, atitinkamai nuolat auga maisto produktų bei pašarų poreikis, todėl globaliu mastu iškyla būtinybė ne tik didinti augalų veislių produktyvumą, bet ir pritaikyti žemdirbystei iki šiol neišnaudotus sausringus ar druskingus dirvožemius (Hussain *et al*., 2009; Godfray *et al.*, 2010).

Drėgmės trūkumas ir dirvožemių druskingumas yra vieni pagrindinių neigiamų abiotinių veiksnių. Dėl klimato kaitos pastaruoju metu fiksuojamas didesnis ekstremalių klimato reiškinių dažnis, o ateities klimato kaitos modeliai numato augančias oro temperatūras, taigi ir augančią sausrų riziką dėl mažėjančio kritulių kiekio, didėjančio garavimo, arba šių faktorių sąveikos (Dai, 2012; Sherwood & Fu, 2014).

Poliploidiniai augalai dažnai yra atsparesni abiotiniams stresams, tokiems kaip sausra ar padidintas dirvožemio druskingumas (Xue *et al*., 2015; Godfree *et al*., 2017), todėl yra adaptyvesni ir geriau geba prisitaikyti augti įvairiose ekologinėse zonose (Blanc & Wolfe, 2004; Sattler *et al*., 2016), tačiau taip pat esama tyrimų, kurių rezultatai rodo, kad būtent diploidiniai augalai yra atsparesni stresams (Sugiyama, 2006; Balocchi & López, 2009; Helgadóttir *et al*., 2018), arba skirtumai tarp skirtingo ploidiškumo grupių yra nedideli (Kemesyte *et al*., 2017). Tokiuose tyrimuose naudojamos veislės ar linijos skiriasi ne tik ploidiškumu, bet ir genetine kilme, todėl sudėtinga atsakyti, ar nustatomi skirtumai buvo nulemti chromosomų skaičiaus pokyčio, ar skirtingų atsparumą stresiniams veiksniams lemiančių genų alelių.

Tyrimų tikslas. Įvertinti gausiažiedės vienametės svidrės (*Lolium muliflorum* ssp. *multiflorum*) diploidinių ir tetraploidinių veislių bei indukuotų tetraploidinių linijų su derlingumu susijusius požymius bei atsparumą abiotiniams veiksniams ir palyginti su atsaku į abiotinius stresus susijusių genų ekspresiją diploidiniuose ir indukuotuose tetraploidiniuose augaluose.

Tyrimų uždaviniai:

1. Nustatyti optimalų poveikio mitozės inhibitoriais metodą ir indukuoti tetraploidines linijas dešimtyje diploidinių veislių.

2. Įvertinti diploidinių ir tetraploidinių linijų bei veislių su derlingumu susijusius požymius ir atsparumą ligoms lauko eksperimente.

3. Įvertinti diploidinių ir tetraploidinių linijų ir veislių atsparumą druskingumui dygimo ir vegetatyvinio augimo tarpsniuose.

4. Įvertinti diploidinių ir tetraploidinių linijų ir veislių atsaką į drėgmės trūkumą kontroliuojamo klimato sąlygomis.

5. Įvertinti diploidinių veislių ir indukuotų tetraploidinių linijų su streso atsaku susijusių kandidatinių genų ekspresiją drėgmės trūkumo streso metu.

Ginamieji teiginiai:

1. Ploidiškumo lygis turi įtaką gausiažiedės vienametės svidrės atsakui į drėgmės trūkumą.

2. Ploidiškumo lygis ir antiradikalinis aktyvumas turi įtaką gausiažiedės vienametės svidrės atsakui į padidintą druskingumą.

3. Su streso atsaku susijusių genų ekspresijos lygio pokytis indikuotų tetraploidinių linijų augaluose susijęs su jų aukštesniu tolerantiškumu drėgmės trūkumui, lyginant su tėvinėmis diploidinėmis veislėmis.

Mokslinio darbo naujumas

Atsakas į sausros ir padidinto druskingumo stresą *Lolium* gentyje yra plačiai tyrinėjamas, tačiau didžioji dauguma tyrimų nukreipti į daugiametes arba gausiažiedes dvimetes svidres, tuo tarpu duomenų apie gausiažiedės vienametės svidrės atsaką į abiotinius stresus mokslinėje literatūroje yra labai mažai. Šiame darbe nustatytas gausiažiedės vienametės svidrės skirtingo ploidiškumo veislių ir indukuotų tetraploidinių linijų atsakas į sausros ir druskingumo stresus lauko ir kontroliuojamomis sąlygomis, įvertinti skirtingo ploidiškumo grupių fenotipiniai požymiai, atliktas indukuotų tetraploidinių linijų ir tėvinių diploidinių veislių su adiotiniu stresu susijusių genų ekspresijos palyginimas sausros streso metu. Diploidinių veislių ir iš jų indukuotų tetraploidinių linijų palyginimas suteikė unikalią galimybę tikslingai įvertinti ploidiškumo įtaką augalo fenotipiniams požymiams ir streso atsakui, išvengiant skirtingos genetinės kilmės įtakos.

Tyrimo rezultatų aprobavimas

Tyrimų tema buvo parengtos dvi publikacijos žurnaluose, turinčiuose citavimo indeksą duomenų bazėje Clarivate Analytics Web of Science, 1 publikacija – knygos skyriuje. Tyrimų rezultatai pristatyti 4 konferencijose.

Disertacijos turinys ir apimtis

Daktaro disertacija parengta anglų kalba. Ją sudaro įvadas, literatūros apžvalga, tyrimo objektas ir metodai, rezultatai ir diskusija, išvados, literatūros sąrašas, mokslinių publikacijų disertacijos tema sąrašas. Disertacijos apimtis – 142 puslapiai, panaudoti 288 literatūros šaltiniai, disertacija iliustruota 18 paveikslų ir 12 lentelių.

TYRIMŲ METODAI

Gausiažiedės vienametės svidrės tetraploidų indukcija

Laboratoriniai tyrimai buvo atlikti Genetikos ir fiziologijos laboratorijoje, Žemdirbystės institute, Lietuvos agrarinių ir miškų mokslų centre. Tetraploidinės linijos buvo indukuotos iš 10 skirtingos kilmės gausiažiedės vienametės svidrės diploidinių veislių remiantis modifikuotu Pašakinskienė (2000) metodu, naudojant skirtingų koncentracijų kolchicino ir amiprofosmetilo (APM) tirpalus bei skirtingas poveikio trukmes. Išgyvenusių augalų ploidiškumas nustatytas tėkmės citometru Partec PA (Partec Gmbh, Vokietija) bei skaičiuojant chromosomas šaknies meristeminių ląstelių preparatuose.

Diploidinių ir tetraploidinių veislių bei indukuotų tetraploidinių linijų lauko eksperimentas

Lauko eksperimentas buvo vykdomas Lietuvos agrarinių ir miškų mokslų centro filialo Žemdirbystės instituto Žolių selekcijos skyriaus sėjomainos laukuose (55°40’ N, 23°87’ E), kuriuose vyrauja neutralios reakcijos (pH – 7,2), vidutinio humusingumo (1,74 %), fosforingi (175 mg kg^{-1}) ir kalingi (157 mg kg^{-1}), vienodos granuliometrinės sudėties giliau karbonatiniai sekliau glėjiški vidutinio sunkumo priemolio rudžemiai (RDg8-k2) (Endocalcari – Epihypogleyic Cambisols CMg-p-w-can). 2017 m. augynai įrengti gegužės 25 d., 2018 m. – gegužės 9 d. Kiekvienos linijos/veislės sėta po 20 augalų, lizdiniu būdu 50 x 50 cm atstumais, 3 pakartojimais, išdėstant atsitiktine tvarka. Prieš sėją tręšta mineralinėmis trąšomis $N_{30}P_{50}K_{70}$, po pjūčių išbertos azoto trąšos – 45 kg ha^{-1} veiklios medžiagos. Augalams esant plaukėjimo tarpsnyje (BBCH 59) atlikti augalų aukščio (cm), vėliavinių lapų ploto (cm^2) ir žiedynų ilgio (cm) matavimai. Vėliavinio lapo ploto nustatymui iš kiekvieno pakartojimo imta 10 augalų po 3 lapus. Matavimai atlikti naudojant programą ImageJ. *Puccinia coronata* pažeidimai vertinti vizualiai (1–9 balų sistema) pradėjus plisti patogenui. Sausųjų medžiagų derliaus nustatymui augalai buvo pjaunami plaukėjimo metu, džiovinami iki pastovios masės (100 °C temperatūroje) ir pasveriami.

Drėgmės trūkumo stresas kontroliuojamo klimato sąlygomis

Šiame eksperimente buvo tiriamos 8 diploidinės veislės, iš jų indukuotos tetraploidinės linijos ir 3 tetraploidinės veislės. Sėklos buvo sudaigintos Petri lėkštelėse ant drėgno filtrinio popieriaus ir persodintos į plastikinius indelius (9 cm diametro, 8 cm aukščio) su smėlingu substratu (54 % kompostas, 32 % smėlis, 14 % durpės), po 5 augalus indelyje, 4 pakartojimais. Augalai buvo auginami 3 savaites fitototrone PlantMaster (CLF Plant Climatics Gmbh, Vokietija) palaikant 16/8 val. fotoperiodą, 24/18 °C, 60 % oro drėgmę. Drėgmės trūkumo streso eksperimentas buvo vykdomas 5 dienas,jo metu kiekvienas indelis buvo laistomas po 10 ml vandens tuo pačiu paros

metu. Kiekvieną dieną buvo fiksuojamas lapų augimo greitis. Po 5 dienų vizualiai įvertintas augalų suvytimas (1 – nėra vytimo požymių, 6 – stiprus suvytimas). Po drėgmės trūkumo streso buvo indukuotas sausros stresas visiškai nelaistant, po 5 d. stresas buvo nutrauktas augalų išgyvenamumo įvertinimui. Eksperimentas buvo pakartotas 3 kartus.

Santykinio vandens kiekio (SVK) ir chlorofilo fluorescencijos (Fv/Fm) nustatymas

SVK buvo nustatytas drėgmės trūkumo eksperimento pabaigoje. Augalų lapai buvo pasverti (ŽM), panardinti į distiliuotą vandenį 6 val., po to pasverti (BM) ir išdžiovinti 70 °C temperatūroje iki pastovios masės (SM). SVK apskaičiuotas pagal formulę SVK (%) = [(ŽM - SM) / (BM - SM)] × 100. Fv/Fm išmatuotas chlorofilo fluorescencijos matuokliu OS30p+ (Opti-Sciences, Inc. JAV).

Antiradikalinio aktyvumo ir bendro fenolių kiekio nustatymas

Augalų antiradikalinis aktyvumas drėgmės trūkumo streso pabaigoje nustatytas laisvųjų radikalų surišimo DPPH metodu pagal Brand-Williams *et al*. (1995) metodiką. Antiradikalinis aktyvumas buvo išreikštas trolokso ekvivalentu gramui sausos masės. Bendro fenolių kiekio nustatymui atlikti matavimai Genesys-10 UV / VIS spektrofotometru (Thermo Spectronic, Rochester, JAV). Bendras fenolinių junginių kiekis buvo išreikštas galo rūgšties ekvivalentu GRE (mg g^{-1}). Antiradikalinio aktyvumo ir bendro fenolių kiekio matavimai atlikti Lietuvos agrarinių ir miškų mokslo centro filialo Sodininkystės ir daržininkystės instituto Biochemijos ir technologijos laboratorijoje.

Atsako į druskingumo stresą nustatymas sėklų dygimo ir daigų tarpsniuose

Sėklų dygimo tyrimams po 50 kiekvienos tiriamosios veislės ir indukuotų tetraploidų linijų sėklų buvo išdėliota Petri lėkštelėse ant filtrinio popieriaus 3 pakartojimais. Filtrinis popierius buvo sudrėkintas distiliuotu vandeniu (kontrolė) ir skirtingos koncentracijos NaCl tirpalu. Sudygusių sėklų skaičius nustatomas kiekvieną dieną, iš viso 10 dienų laikotarpiu. Eksperimentas pakartotas 3 kartus. Pagal gautus duomenis apskaičiuotas dygimo procentas (DP), dygimo indeksas (DI), vidutinis sudygimo laikas (VDL) ir laikas, per kurį sudygo 50 % sėklų (T50).

Atsako į druskingumo stresą daigų tarpsnyje nustatymui tiriamųjų veislių ir indukuotų tetraploidinių linijų sėklos buvo pasėtos plastikiniuose indeliuose (9 cm diametras, 8 cm aukštis), užpildytuose perlito-vermikulito 1:1 mišiniu. Augalai buvo auginami 3 savaites 25 ± 2 °C, 16/8 h fotoperiodu. Druskingumo stresas indukuotas 10 dienų naudojant 500 mM NaCl tirpalą, kiekvieną dieną tuo pačiu metu buvo matuojamas lapų augimas. Po 10 dienų buvo nustatytas SVK, antiradikalinis aktyvumas ir bendras fenolių kiekis. Eksperimentas kartotas 3 kartus.

Genų ekspresijos analizė

Šiam tyrimui buvo pasirinktos diploidinės veislės 'Varpė' ir 'Magloire' bei iš jų indukuotos tetraploidinės linijos. Augalai auginti ir drėgmės trūkumo stresas sukeltas kaip aprašyta aukščiau. Mėginiai genų ekspresijos tyrimams surinkti pirmą, trečią ir penktą streso dieną. RNR išskirta naudojant GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, Lietuva) pagal gamintojo instrukcijas. RNR koncentracija įvertinta naudojant NanoDrop 2000 (Thermo Fisher Scientific, JAV), cDNR sintezė atlikta su RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lietuva) pagal gamintojo instrukcijas. Amplifikacija atlikta naudojant PowerUp SYBR Green Master Mix (Applied Biosystems, UK) ir 7500 Fast Real Time PCR System (Applied Biosystems, JAV).

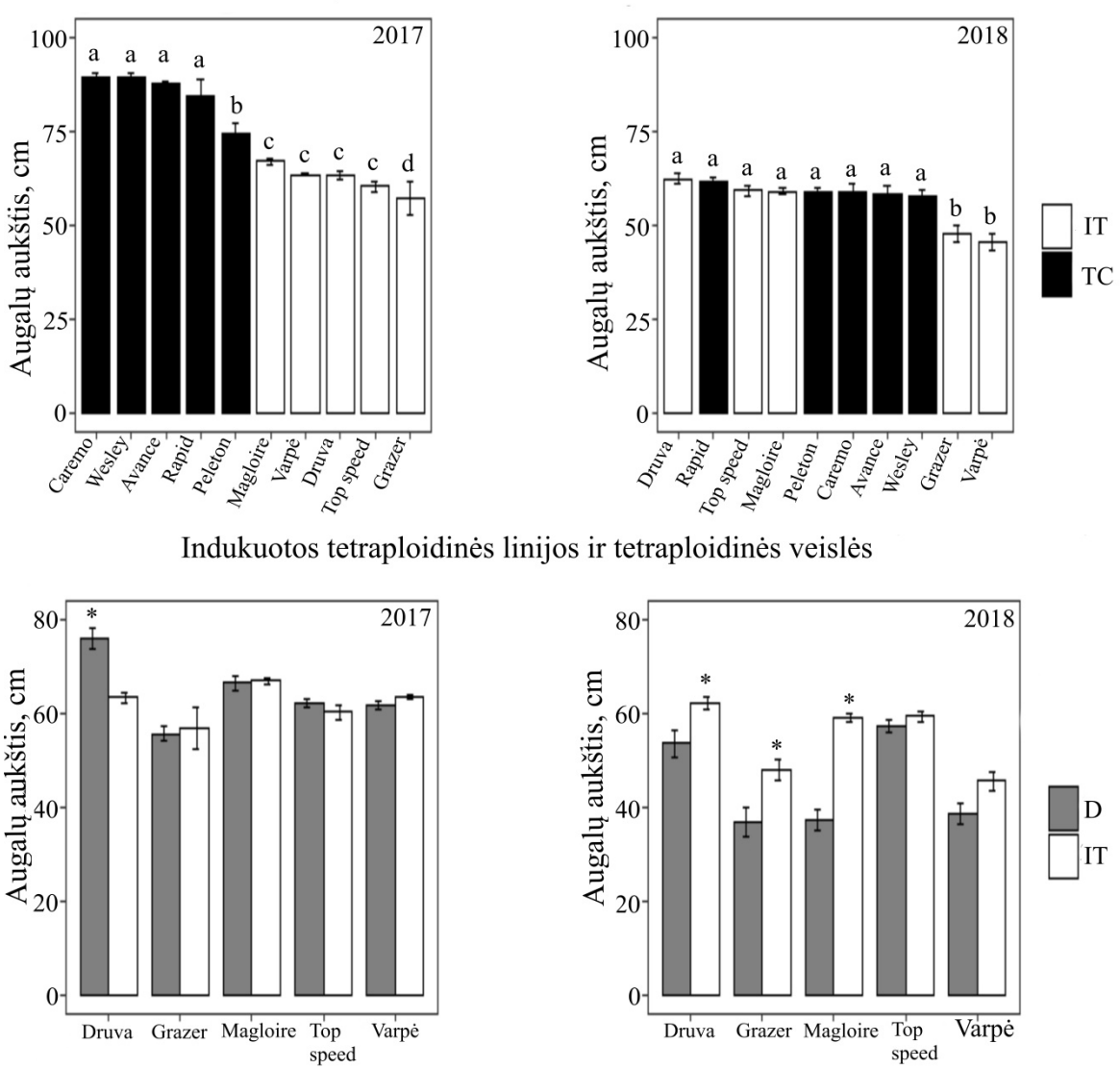
REZULTATAI IR JŲ APTARIMAS

Tetraploidų indukcija

Mitozės inhibitorių efektyvumas indukuojant tetraploidus priklauso nuo jų toksiškumo, optimalios koncentracijos ir poveikio laiko bei genotipinio atsako Tetraploidai buvo indukuoti naudojant du mitozės inhibitorius, kolchiciną ir amiprofosmetilą (APM), tačiau APM poveikis augalams buvo toksiškesnis nei kolchicino net ir mažesnėmis koncentracijomis, augalų išgyvenamumas naudojant kolchiciną buvo aukštesnis nei naudojant APM. Aukščiausias tetraploidų indukcijos efektyvumas gausiažiedėje vienametėje svidrėje buvo pasiektas naudojant poveikį kolchicinu (10 mM, 3 h, arba 8 mM, 4 h).

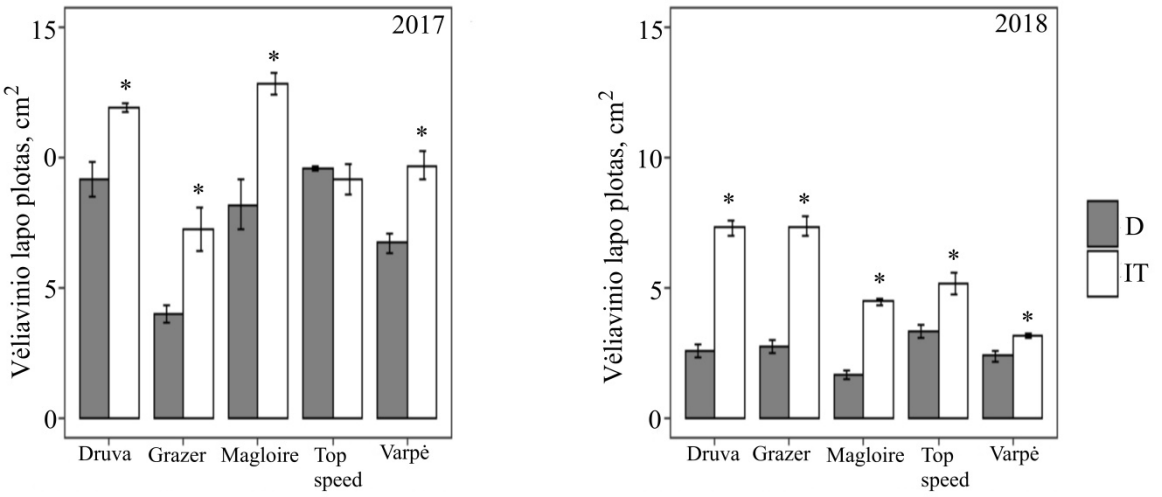
Diploidinių ir tetraploidinių veislių bei indukuotų tetraploidinių linijų lauko eksperimentas

Augalų aukštis. Pirmaisiais tyrimų metais statistiškai patikimų skirtumų tarp diploidinių veislių ir indukuotų tetraploidinių linijų nustatyta nebuvo, išskyrus veislę 'Druva', kurios augalai buvo reikšmingai aukštesni nei atitinkamos tetraploidinės linijos. Tetraploidinių veislių augalai buvo reikšmingai aukštesni už diploidinių veislių ir tetraploidinių linijų augalus. Antraisiais (2018) tyrimų metais, kurie buvo sausringi ir pasižymėjo aukštesne vidutine oro temperatūra, daugumos tetraploidinių linijų augalai buvo aukštesni nei tėvinių diploidinių veislių augalai (1 pav.).



1 pav. *Diploidinių veislių (D), indukuotų tetraploidinių linijų (IT) ir tetraploidinių veislių (TC) augalų aukštis 2017–2018 m. Skirtingos raidės žymi statistiškai patikimus skirtumus tarp tetraploidinių linijų ir veislių, * žymi statistiškai patikimus skirtumus tarp diploidinių linijų ir iš jų indukuotų tetraploidinių linijų ($p < 0,05$)*

Vėliavinio lapo plotas, ilgis ir žiedynų ilgis. Tetraploidinių grupių (indukuotų linijų ir veislių) augalų vėliavinio lapo plotas buvo reikšmingai didesnis lyginant su diploidinėmis veislėmis abejais tyrimų metais. Sausringos sąlygos 2018 m. neigiamai paveikė abiejų citotipų lapų plotą – jis buvo mažesnis nei 2017 m. (2 pav.).



***2 pav**. Diploidinių veislių (D) ir indukuotų tetraploidinių linijų (IT) augalų vėliavinio lapo plotas 2017–2018 m. * žymi statistiškai patikimus skirtumus tarp diploidinių veislių ir iš jų indukuotų tetraploidinių linijų ($p < 0,05$)*

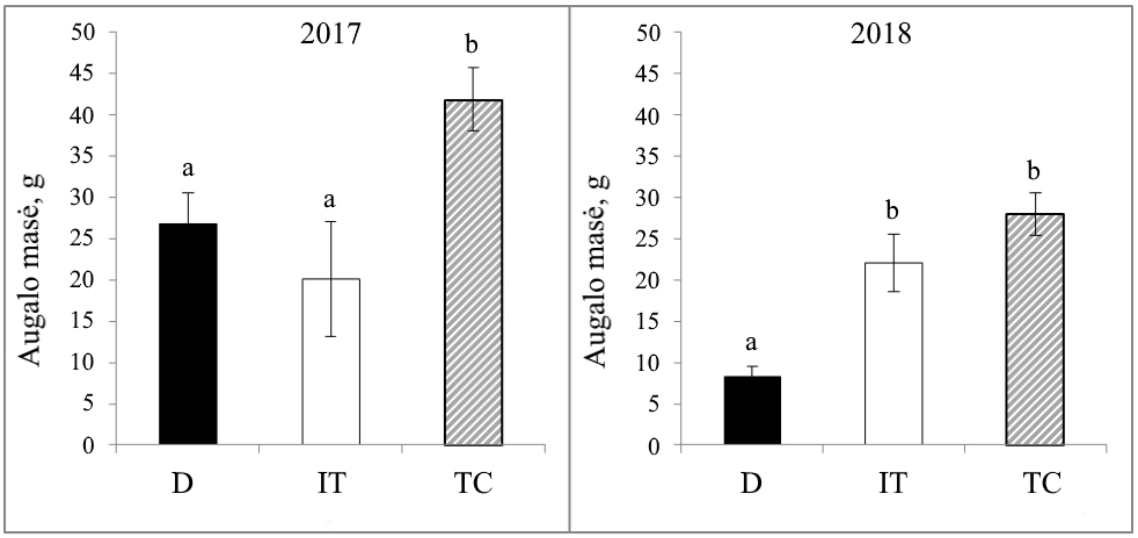
Vėliavinio lapo ilgis taip pat buvo paveiktas sausros: 2017 m. statistiškai patikimų vėliavinio lapo ilgio skirtumų tarp daugumos diploidinių veislių ir tetraploidinių linijų nenustatyta, tačiau 2018 m. daugumos indukuotų tetraploidinių linijų augalų vėliaviniai lapai buvo ilgesni nei tėvinių diploidinių veislių. Indukuotų tetraploidinių linijų augalų žiedynai buvo ilgesni nei diploidinių veislių abejais tyrimų metais, išskyrus veislę 'Top Speed', tačiau 2018 m. šie skirtumai buvo dar ryškesni.

Indukuotų tetraploidinių linijų genomo epigenetiniai pokyčiai ir genomo nestabilumas, sukelti mutageninio kolchicino poveikio, galėjo turėti įtaką lauko tyrimų rezultatams 2017 m. Tuo tarpu fenotipinių požymių skirtumai tarp indukuotų tetraploidinių linijų ir tėvinių diploidinių linijų, gauti antraisiais tyrimų metais, rodo kad poliploidiškumas galėjo nulemti didesnį tetraploidinių linijų atsparumą sausrai lauko sąlygomis.

Sausųjų medžiagų derlius. Derlingumas yra vienas svarbiausių požymių pašarinių augalų selekcijoje. Tetraploidinės veislės formavo didesnę augalo sausųjų medžiagų masę (SMM) abejais tyrimų metais palyginus su diploidinėmis veislėmis, tuo tarpu indukuotų tetraploidinių linijų augalų SMM 2017 m. statistiškai nesiskyrė nuo diploidinių veislių. 2018 m. dėl sausros poveikio visų tiriamų grupių SMM buvo mažesnė nei 2017 m., tačiau išryškėjo skirtumai tarp abiejų tetraploidinių grupių ir diploidinių veislių (3 pav.). Indukuotų tetraploidinių linijų SMM buvo didesnė nei diploidinių veislių ir statistiškai nesiskyrė nuo tetraploidinių veislių. Tai rodo, kad ploidiškumas turėjo įtakos augalų SMM sausros sąlygomis.

Vainikuotųjų rūdžių infekcija. 2018 m. vainikuotosios rūdys (*Puccinia coronata*) paveikė visas veisles ir indukuotas linijas lauko tyrimuose, nepriklausomai nuo ploidiškumo lygio. Tarp tiriamųjų veislių ir linijų išsiskyrė 'Magloire' – tiek veislės, tiek iš jos indukuotos tetraploidinės

linijos augalai buvo atsparesni infekcijai, tuo tarpu iš 'Top speed' indukuota tetraploidinė linija buvo jautriausia rūdims.



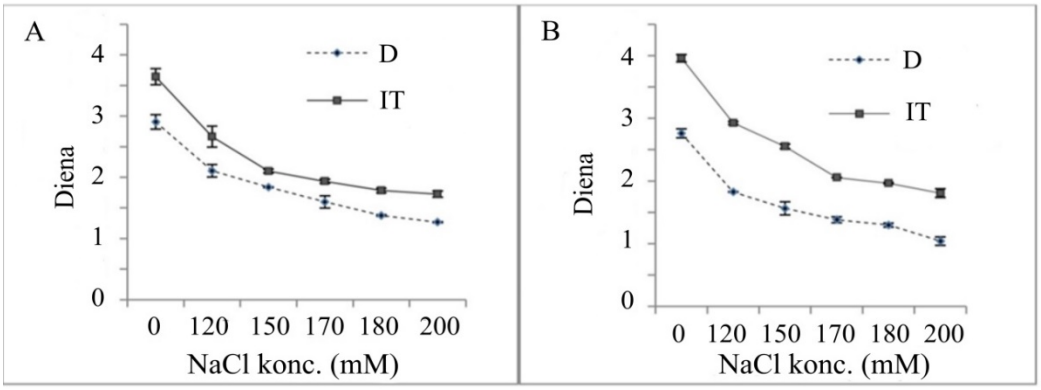
***3 pav**. Diploidinių veislių (D), indukuotų tetraploidinių linijų (IT) ir tetraploidinių veislių (TC) augalo vidutinė sausųjų medžiagų masė 2017–2018 m. Skirtingos raidės žymi statistiškai patikimus skirtumus ($p < 0,05$)*

Augalų atsakas į drėgmės trūkumo stresą kontroliuojamomis sąlygomis

Sausros sukeliami nuostoliai priklauso nuo drėgmės trūkumo intensyvumo ir streso trukmės. Šiame eksperimente buvo įvertintas diploidinių ir tetraploidinių veislių bei indukuotų tetraploidinių linijų fenolinių junginių kiekis ir antiradikalinis aktyvumas po drėgmės trūkumo streso bei augalų gebėjimas atželti po visiškos sausros periodo. Indukuotų tetraploidinių linijų augaluose nustatytas statistiškai patikimai aukštesnis antiradikalinis aktyvumas ir didesnis fenolių kiekis lyginant su tėvinėmis diploidinėmis veislėmis. Taip pat tetraploidinės linijos ir tetraploidinės veislės geriau išgyveno ir greičiau atžėlė po intensyvios sausros streso. Stipri koreliacija nustatyta tarp išgyvenamumo po intensyvios sausros ir antiradikalinio aktyvumo ($r = 0,79$, $p \leq 0,05$) bei bendro fenolių kiekio ($r = 0,72$, $p \leq 0,05$) po drėgmės trūkumo streso. Šie rezultatai leidžia daryti prielaidą, kad regionuose, dažnai patiriančiuose sausras vegetacijos periodu, tikslinga auginti poliploidines veisles, nes tetraploidiniai augalai geriau išgyvena ir vėliau atželia sausrai pasibaigus.

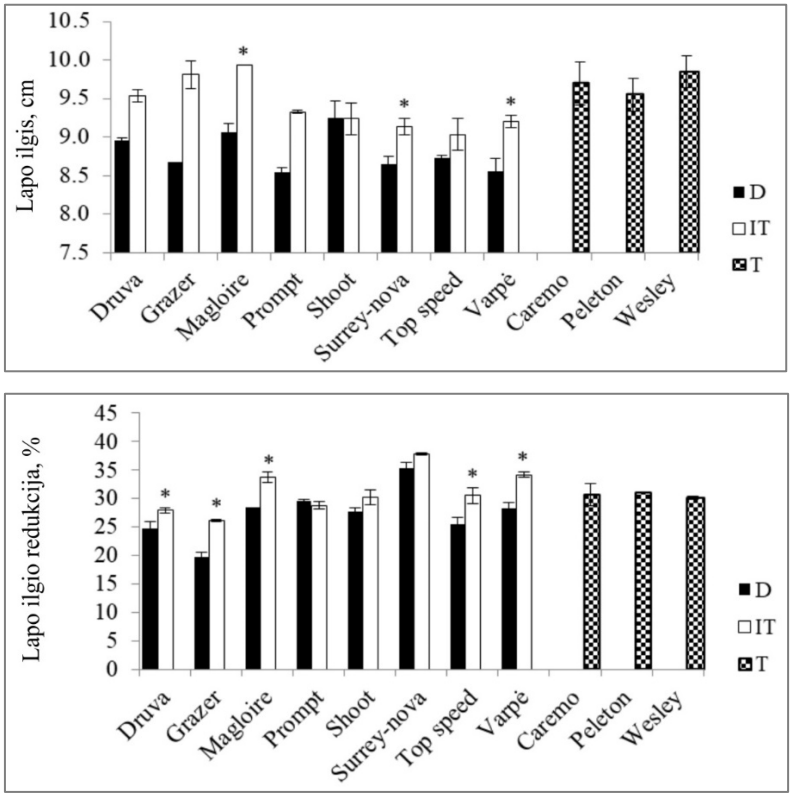
Augalų atsakas į druskingumo stresą kontroliuojamomis sąlygomis

Poveikis sėklų dygimo tarpsnyje. Druskingumo stresas vėlino gausiažiedės vienametės svidrės sėklų dygimą arba visiškai jį inhibavo abejose ploidiškumo grupėse ir skirtinguose genotipuose (4 pav.), tačiau poveikis tetraploidinėms linijoms buvo mažesnis nei diploidinėms veislės, remiantis paskaičiuotomis dygimo indekso ir T50 vertėmis. Nustatyta stipri koreliacija tarp NaCl koncentracijos ir dygimo inhibicijos ($r = 0,86$, $p \leq 0,01$).



***4 pav**. Diploidinių veislių (D) ir indukuotų tetraploidinių linijų (IT) dygimo indeksas skirtingos koncentracijos NaCl tirpaluose. A – 'Magloire', B – 'Varpė'.*

Poveikis daigų tarpsnyje. Druskingumo stresas neigiamai veikė augalų lapų augimą visose tiriamosiose veislėse ir linijose. Tetraploidinės linijos ir veislės formavo ilgesnius lapus negu diploidinės veislės, nepaisant to, kad jose nustatytas didesnis lapo ilgio sumažėjimas lyginant su kontrolinės grupės augalais. Atsako į druskingumo stresą stiprumas taip pat priklausė ir nuo genotipo (5 pav.).



***5 pav**. Diploidinių veislių (D), indukuotų tetraploidinių linijų (IT) ir tetraploidinių veislių (T) daigų atsakas į druskingumo stresą (500 mM NaCl). A – augalų lapų ilgis po 10 d., B – augalų lapų ilgio sumažėjimas lyginant su kontrole. * žymi statistiškai patikimus skirtumus tarp diploidinių veislių ir iš jų indukuotų tetraploidinių linijų ($p < 0,05$)*

Druskingumo streso poveikis augalų antiradikaliniam aktyvumui. Antiradikalinis aktyvumas buvo nustatytas tetraploidinėse linijose ir tėvinėse diploidinėse veislėse po 10 d. druskingumo streso poveikio. Indukuotų tetraploidų linijos pasižymėjo aukštesniu antiradikaliniu aktyvumu, lyginant su diploidinėmis veislėmis, išskyrus veislę 'Prompt' (1 lentelė). Statisitiškai patikima koreliacija ($p \leq 0,05$) nustatyta tarp antiradikalinio aktyvumo ir dygimo indekso ($r = 0,55$) bei santykinio augalų lapo ilgio sumažėjimo ($r = 0,64$).

1 lentelė. *Druskingumo streso poveikis gausiažiedės vienametės svidrės diploidinių veislių ir indukuotų tetraploidinių linijų augalų santykiniam vandens kiekiui (SVK) lapuose ir antiradikaliniam aktyvumui. Skirtingos raidės žymi statistiškai patikimus skirtumus (p < 0,05)*

Diploidinė veislė/ Tetraploidinė linija	SVK %	Antiradikalinis aktyvumas, kontrolė (µmol TE g^{-1})	Antiradikalinis akatyvumas, 500 mM NaCl, 10 d. (µmol TE g^{-1})
Varpė	68,8 ± 0,19 g	23,7 ± 3,22 de	35,0 ± 0,78 fg
Varpė-4x	75,9 ± 0,49 de	35,4 ± 1,34 a	47,3 ± 0,95 ab
Druva	80,2 ± 1,18 c	32,7 ± 1,01 ab	36,6 ± 1,35 g
Druva-4x	71,8 ± 0,5 f	29,6 ± 1,35 bc	44,3 ± 0,98 bc
Magloire	69,1 ± 0,31 g	33,4 ± 1,81 ab	41,5 ± 2,63 cd
Magloire-4x	80,6 ± 1,01 c	33,2 ± 1,15 ab	49,2 ± 0,11 a
Grazer	75,9 ± 1,43 de	23,0 ± 0,15 e	28,8 ± 2,28 h
Grazer-4x	74,4 ± 0,93 ef	24,7 ± 0,55 de	39,0 ± 1,97 de
Surrey Nova	87,6 ± 1,02 a	27,3 ± 0,54 cde	32,8 ± 1,34 fg
Surrey Nova-4x	83,5 ± 1,01 b	23,3 ± 2,87 de	42,7 ± 2,33 c
Prompt	73,7 ± 0,21 ef	27,8 ± 0,72 cd	36,5 ± 1,77 ef
Prompt-4x	81,0 ± 1,01 bc	28,1 ± 0,02 cd	38,8 ± 2,35 de
Top speed	77,4 ± 0,69 d	27,5 ± 0,50 cde	35,3 ± 1,45 efg
Top speed-4x	88,1 ± 0,63 a	24,0 ± 0,78 de	41,3 ± 1,23 cd
Shoot	74,0 ± 1,00 ef	30,0 ± 0,52 bc	35,7 ± 0,55 efg
Shoot-4x	80,4 ± 0,36 c	32,8 ± 1,14 ab	43,2 ± 0,89 c

Su sausros stresu susijųsių genų ekspresija gausiažiedės vienametės svidrės diploidinėse veislėse 'Varpė' ir 'Magloire' bei iš jų indukuotose tetraploidinėse linijose

Dehidrino *(Dh3)* geno ekspresija. Dehidrinai yra hidrofiliniai baltymai, priklausantys II LEA grupei. Dehidrinai akumuliuojami vėlyvose augalų embriogenezės stadijose ir yra siejami su desikacijos tolerancija (Battaglia *et al.,* 2008; Liu *et al.,* 2017). Pirmąją streso poveikio dieną *Dh3*

ekspresija buvo žema visuose tirtuose augaluose, ji reikšmingai padidėjo trečiąją dieną, o penktąją dieną nustatyta aukščiausias ekspresijos lygis, kuris indikuotuose teraploiduose buvo reikšmingai didesnis nei diploidinėse veislėse.

***HUB1* geno ekspresija.** Ubikvitinas ir į ubikvitiną panašūs (ubiquitine-like) baltymai dalyvauja daugelyje svarbių ląstelinių ir augalo vystymosi reguliavimo procesų (Miura & Hasegawa, 2010). *HUB1* geno ekspresija išaugo penktąją streso dieną abejose ploidiškumo grupėse, tačiau tetraploidiniuose augaluose ji buvo aukštesnė nei diploidiniuose.

Superoksido dismutazės *(Cu/Zn SOD),* katalazės *(CAT),* askorbato peroksidazės *(APX),* gvajakol-peroksidazės *(POD)* ir glutationo peroksidazės *(GPX)* genų ekspresija. Šių genų koduojami baltymai dalyvauja antioksidaciniuose metaboliniuose procesuose. Genai yra aktyvuojami sausros streso metu ir yra gyvybiškai svarbūs reaktyvių deguonies formų (RDF) detoksikacijoje. Cu/Zn SOD dalyvauja (O_2^-) konversijoje į H_2O_2 ir O_2 ir yra vienas pirmųjų genų, aktyvuojamų esant oksidaciniam stresui (Liu & Huang, 2000). Susidaręs H_2O_2 taip pat yra toksiškas augalų ląstelėms, todėl kiti baltymai, tokie kaip CAT, POD, APX, ir GPX dalyvauja jo konversijoje į vandenį. Tyrimuose nustatyta padidėjusi visų šių su oksidaciniu stresu susijusių genų ekspresija, ypač indukuotuose tetrpaloidiniuose augaluose.

Pirolino 5-karboksilato reduktazės *(LpP5CR)* geno ekspresija. Pirolino 5-karboksilato reduktazė katalizuoja Δ1-pirolino-5-karboksilato konversiją į proliną. Eksperimento metu *LpP5CR* geno ekspresija išaugo, didžiausias pokytis nustatytas 'Varpės' indukuotos tetraploidinės linijos augaluose penktąją drėgmės trūkumo streso dieną.

IŠVADOS

1. Gausiažiedės vienametės svidrės tetraploidiniai augalai gali būti indukuoti naudojant kolchiciną arba amiprofosmetilą (APM). Tetraploidų indukcijos efektyvumas priklauso nuo panaudoto mitozės inhibitoriaus koncentracijos, poveikio trukmės bei toksiškumo. Poveikis kolchicino 10 mM tirpalu 3 valandas arba 8 mM tirpalu 4 valandas buvo efektyviausias gausiažiedės vienametės svidrės tetraploidų indukcijos metodas.

2. Didesnis ploidiškumo lygis turėjo teigiamą įtaką augalų morfologiniams požymiams ir sausųjų medžiagų derliui indukuotų tetraploidinių linijų ir tėvinių diploidinių veislių lauko tyrimuose, ypač drėgmės trūkumo sąlygomis.

3. Padidėjęs druskingumas reikšmingai mažino gausiažiedės vienametės svidrės sėklų dygimą. Indukuotos tetraploidinės linijos pasižymėjo aukštesniu dygimo indeksu ir mažesniu T50 įverčiu lyginant su tėvinėmis diploidinėmis veislėmis. Vegetatyvinio augimo tarpsnyje druskingumo streso sąlygomis indukuotų tetraploidinių linijų augalai pasižymėjo aukštesniu antiradikaliniu aktyvumu nei diploidinės veislės. Tai rodo, kad poliploidiškumas turi įtakos tolerantiškumui druskingumo stresui.

4. Chromosomų skaičiaus padvigubinimas turėjo įtakos gausiažiedės vienametės svidrės atsakui į sausros stresą: indukuotų tetraploidinių linijų augaluose nustatytas aukštesnis antiradikalinis aktyvumas bei didesnis fenolinių junginių kiekis lyginant su diploidinėmis veislėmis drėgmės trūkumo streso metu kontroliuojamomis sąlygomis. Nustatyta reikšminga koreliacija tarp antiradikalinio aktyvumo ir atžėlimo po sausros streso.

5. Su atsaku į sausros stresą susijusių genų ekspresijos lygis skyrėsi tarp indukuotų tetraploidinių linijų ir tėvinių diploidinių veislių – tetraploidinėse linijose nustatytas aukštesnis ekspresijos lygis drėgmės trūkumo sąlygomis, galintis lemti didesnį tetraploidų atsparumą šiam stresui.

MOKSLINIŲ PUBLIKACIJŲ SĄRAŠAS

Straipsniai recenzuojamuose mokslo leidiniuose, turinčiuose citavimo indeksą „*Clarivate Analytics Web of Science*" duomenų bazėje

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Olakunle Kelvin Akinroluyo was born in Nigeria on the 16th of December 1984. He completed his secondary school at the Federal University of Technology Akure, staff secondary school in 2001. Kelvin attended Olabisi Onabanjo University and studied biochemistry from 2003 to 2008 and bagged his Bachelor of Science degree. He then obtained his Masters degree in business administration from Anglia Ruskin University in England from 2011 to 2012. His thirst for science was not quenched. He studied agrobiotechnogy at Vytautas Magnus University from 2013 to 2015 and obtained his Masters degree. He was also a doctoral student at the Lithuanian research Centre for Agriculture and Forestry from 2015 to 2019.

Olakunle Kelvin AKINROLUYO

EFFECT OF PLOIDY LEVEL ON PLANT ABIOTIC STRESS RESPONSE IN WESTERWOLTHS RYEGRASS

Doctoral dissertation

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