1 **Comparative physiological and biochemical mechanisms in Di, Tri, and**
2 **Tetraploid Watermelon (*Citrullus lanatus* L.) grafted by Branches**
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11   **Abstract**

12   Polyploid seeds production is laborious, complicated, and costly work. Tetraploid and

13   triploid plants produce a fewer number of seeds/fruit, and triploid embryos are fairly

14   weak, covered with a more hardened seed coat as compared to diploid seeds. Here we

15   investigated the interactive effect of new grafting technique of polyploid watermelon

16   scion onto rootstock on plants' survival rate, biochemical, and hormones contents. In

17   this study, three different branches, apical meristem (AM), branch with 1 node (1N),

18   and 2 nodes (2N) from di, Tri, and tetraploid watermelon plants, were used as scion and

19   grafted onto squash rootstock. The results showed highly significant differences

20   between polypoid watermelon when 1N using as a scion, tetraploid showed maximum

21   survival rates, higher contents of hormones, and antioxidants (AOX) activities,

22   compared to diploid, these may be the possible reasons for high compatibility in

23   tetraploid and degrading the grafting zone in diploid. RTq-PCR results confirm that the

24   expression of genes linked to compatibility is consistent with the hormonal and AOX

25   activities. This study provides an alternative and economical approach to produce more

26   tetraploid and triploid plants for breeding or seeds production by using branches as

27   scions.

28   **Keywords:** watermelon, polyploidy, grafting, branches

30

1. Introduction

Watermelon is the fifth most-consumed flesh fruit worldwide and occupies 7% of the cultivated land for fruits and vegetables (FAOSTAT 2018). Seedless watermelons are the most desirable watermelon cultivars presently available to consumers and command a high price and more excellent quality than seeded watermelon [1]. Seedless watermelons are produced by crossing a tetraploid seed parent with a diploid pollen parent [2]. Tetraploid induction can be done by different methods like applying aqueous chemicals (colchicine, Oryzalin, and Dinitroaniline) solution to the growing apex of diploid seedlings or by soaking diploid seeds before germination [3]; these chemicals are toxic to the plant material treated, so the percentage of success was low [4]. However, seedless watermelon production has been hampered by high seed cost and poor seed germination. High seed cost has generally been because of difficulties in obtaining a sufficient number of tetraploid individuals and the low number of seeds only 5-20 seeds/fruit as compared to diploid fruits (600 seeds/fruit). It is important to find a more economical way to increase the number and quality of triploid seeds [5-13]

Grafting has a very long history, and evidence of its use has been found in ancient civilizations, e.g., in 1560 BC in China, as discussed by Aristotle (384-322 BC) [14]. Recently, the practice of grafting watermelons has become popular in the world because it can improve disease resistance, tolerance to abiotic stress, fruit quality, and plant size [15-18]. In recent years, splice grafting is one of the most commonly used grafting methods, has achieved about 90% of grafting success, because it is the fastest, most efficient [19-23].

Graft compatibility can be defined as a complex biochemical and structural process that begins with an initial wound response, followed by a callus formation, the creation of a continuous cambium, and a functional vascular system between scion and rootstock [24]. All of these steps can determine the future of a grafted plant [25]. However, still now no general rules, on how these treatments affect incompatibility responses [26,27].

In fruit trees, graft incompatibility occurs due to the accumulation of phenolic compounds [28]. [29] found that reduced auxin and lignification was associated with high phenols in incompatible grafts and may suggest weak graft unions, or reactive oxygen species [30-33], or hormone imbalance [26,34,35]. IAA, and cytokinin, play an important role in regulating stock–scion interactions [36], and required for vascular bundle regeneration in the graft union [37,38]. Plant hormones are important for

64   vascular formation; auxin is important for the differentiation of vascular tissues and
65   wound healing [15,18,32,33,39-45]. Reactive Oxygen Species (ROS) accumulation leads
66   to cell death, and oxidative damage depends on the balance between the production of
67   antioxidant AOX enzymes and ROS [46,47]. Increase the activity of defense enzymes
68   such as Peroxidase POD, and catalase CAT can scavenge ROS in plants, and improving
69   the resistance of plants to stress [48,49]. POX and CAT convert H_2O_2 to H_2O [50]. So the
70   difference relation between AOX and ROS during the healing process could be used as
71   a rapid mechanism to verify incompatibility [51]. Lignin is abundant in woody plants
72   and primarily contributes strength to the cell wall; so, decreased lignin would be
73   antagonistic to a strong graft union [52].
74   Up to date, the cause and underlying mechanism for grafting incompatibility
75   remain elusive, and there is no report on genes associated with grafting
76   incompatibility [53,54]. More research work needs to be done to fully understand the
77   mechanisms of graft compatibility and incompatibility [41,52].
78   The purpose of this research was to study the effect of genome duplication on graft
79   compatibility, by comparing the factors and parameters which lead to graft
80   compatibility or incompatibility. This study provides information at the molecular and
81   physiological levels for the mechanism compatibility in polyploid watermelons, which
82   should explain the mechanism of compatibility in polyploid watermelon, also to try a
83   new technique for vegetable propagation by using branches as scions, three different
84   branches were taken from mother plants and graft on squash rootstock to study the
85   success of this method. This method will add an option for breeders and seed producers
86   to save time and money using an asexual method to increase plant numbers.
87

2. MATERIALS AND METHODS

2. 1. Plant materials

Seedless watermelons are triploids (3n=33) produced by crossing a tetraploid seed parent with a diploid (2n=22) pollen parent. Tetraploid induction was done by applying colchicine to the growing apex of diploid seedlings. Polyploid seeds for one variety (*mimei*), which is homozygous and genetically stable, and passed the achievement appraisal of the Chinese Department of Agriculture in 1990 and won the second prize of science technology progress of the Department of Agriculture in 1991 [55], were used as a mother plant and squash interspecific hybrid *(xijiaqiangsheng)* which widely used in China as a rootstock, obtained from the polyploid watermelon group - Zhengzhou Fruit research institute (CAAS) China. Tetraploid, triploid, and diploid watermelon and rootstock seeds were sown in seedling cell trays with 32 cells at the intelligent greenhouse of Zhengzhou, Henan province, China. After 50-60 days from the date of transplanting, good and healthy mother plants free of pests and diseases, especially virus-free, were selected for obtaining different types of scions. From the healthy mother plants, suitable branches were chosen for scions. Three types of branches (Figure 1) were taken from mother plants, (a) apical meristem (AM), (b) the branch having one node and one leaf (1N), and (c) the branch has two nodes and one leaf (2N) as mentioned by [10]. The grafting process was performed after 15 and 20 days after rootstock seeds sowing. The splice grafting method was used [19] (Figure S1). Rootstock seedlings were subjected to adaptation before and after grafting to increase the survival rate, as mentioned by [56,57].
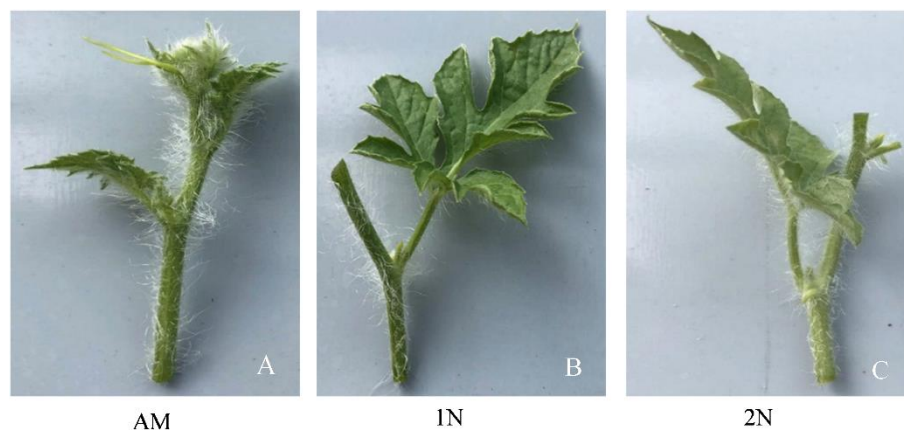
Figure 1. Three types of branches were taken from mother plants to use as scions in di, Tri, and tetraploid watermelon, (A) apical meristem (AM), (B) branch has one node, and one leave (1N), and (C) branch has two nodes (2N)

113   As the survival rate by using one node was higher and had significant differences
114   between Di, Tri and Tetra watermelon, the samples (grafting union) from branch with
115   one node (1N) Were collected at three different stages, (0, 3, and 15 days after grafting)
116   for the determination of hormones, AOX and RNA extraction. Plants were cultivated in
117   the growth chamber and the growth condition was set to 25-30 ºC temperature and 60-
118   85% humidity. The sensor in the center of each experimental plot was used to record
119   data for temperature and humidity (THtool-V151_En; Campbell Scientific Ltd., China).

120   *2.2. Data collection*

121   *2.2.1. Survival rates*

122   Survive rates were investigated after 15 days using the formula [49].

123   $$\text{Survival rate} = \frac{\text{Number of survived grafted plants}}{\text{Total number of grafted plants}} \times 100$$

124   *2.2.2. Detection of Hormones Content by ELISA*

125   Samples were collected from the graft union at 0, 3, and 15 days after grafting (DAG)
126   with three biological replicates. The contents of endogenous Indole-3-acetic acid (IAA)
127   and Zeatin Riboside (ZR) were measured with the Enzyme-Linked Immunosorbent
128   Assay (ELISA) method (College of Agriculture and Biotechnology, China Agricultural
129   University) with three biological replicates for each set of treatments. The
130   determination of hormonal contents was performed as outlined by [36].

131   *2.2.3. Assay for antioxidants enzymes activity and H_2O_2 contents*

132   SOD assay kit/ YX-C-A500 was used for the measurement of superoxide dismutase
133   activity at 560 nm wavelength. CAT assay kit/BC0200 for Catalase assay using 240 nm
134   wavelength, POD assay kit/ YX-C-A502 for peroxidase activity using 570 nm
135   wavelength, and H_2O_2 assay kit/ YX-C-A400 for Hydrogen peroxidase content using
136   415 nm wavelength, (Sino best biological technology co, Ltd, Beijing, China)
137   according to the manufacturer's instructions. Three biological replications from
138   grafting union at 0, 3, and 15 (DAG) from three different plants for each replicate were
139   collected for analysis. The activities of the antioxidant enzymes were expressed as unit
140   U/g FW sample.

141   *2.2.4. Quantification of biochemical at different stages*

142   All experiments were carried out using starch assay kit/ YX-C-C400 for starch content
143   estimation at 620 nm wavelength, lignin assay kit/ YX-C-B636 for lignin content using
144   280 nm wavelength, and phenol assay kit/ YX-C-A507 for total phenols content using

145   760 nm wavelength. (Sino best biological technology co, Ltd, Beijing, China)

146   according to the manufacturer's instructions. Three biological replications from

147   grafting union at 0, 3, and 15 DAG from three different plants for each replicate were

148   collected for analysis.

149   *2.2.5. Identification of genes involved in the compatibility mechanisms.*

150   The protein sequences of genes involved in compatibility mechanisms were

151   downloaded from the watermelon database http://cucurbitgenomics.org/organism/2. A

152   total of 26 genes involved in compatibility mechanisms were identified based on the

153   similarity index between protein sequences. The protein sequences for compatibility

154   mechanisms in watermelon were downloaded from the 97,103 Watermelon Genome

155   Database. MEGA 6 software was used to draw phylogenetic trees of selected genes.

156   The clustalW tool was first used for the alignment. Afterward, the neighbor-joining

157   method, with 1000 bootstrap replicates, was used [58,59]. The phylogenetic trees were

158   constructed using protein sequences of the compatibility mechanisms.

159   *2.2.6. Characteristics and structural analysis of genes responsible for compatibility*

160   *mechanisms in watermelon*

161   Gene names, accession number of genes, genomic lengths, coding sequences (CDS)

162   lengths, protein sizes, and isoelectric points (pIs) and Mw (Da) were downloaded from

163   http://cucurbitgenomics.org/organism/1 and ExPASy http://web.expasy.org/computepi/

164   databases. Gene Structure Display Server (GSDS), a web-based bioinformatics tool,

165   was used to give a structural representation to selected watermelon genes responsible

166   for sugars and organic acids metabolism and transporter genes, including

167   upstream/downstream regions, coding sequences (CDS), and intron numbers were

168   constructed [60].

169   *2.2.7. Isolation of RNA and first-strand cDNA synthesis*

170   Using a Total RNA Kit (Tiangene, China), we extracted the total RNA from the

171   grafting union according to the manufacturer's instructions. Samples from grafting

172   unions were taken at three stages (0, 3, and 15 days after grafting). We detected RNA

173   degradation and contamination on a 1% agarose gel. Using a NanoDrop ND-1000

174   spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100

175   Bioanalyzer (Agilent Technologies, CA, USA), the quantity quality of RNA was

176   checked according to manufacturer's instructions. To carry out quantitative reverse-

177   transcription PCR (RTq-PCR), cDNA was synthesized from RNA with M-MLV
178   Reverse Transcriptase (Promega, USA) and diluted 20 ng/ul.

179   *2.2.8. RTq-PCR expression analysis of genes involved in compatibility mechanisms*
180   Genes linked to the compatibility mechanisms were selected from the watermelon
181   genome database (http://cucurbitgenomics.org/organism/21). To check the expression
182   patterns of selected genes in grafted watermelon at 0, 3, and 15 DAG, quantitative
183   reverse transcription PCR (RTq-PCR) was performed. The entire data were analyzed
184   using the 2-$\Delta\Delta$Ct method [61]. Three independent biological replicates were used for
185   gene expression analysis [62]. Actin *"cla016178"* was used as a reference gene [58].

186   2.2.9. Experimental design and statistical analysis
187   Data were statistically analyzed using analyses of variance (ANOVA), with the Stat
188   soft statistical package MSTATC software program (Michigan State University, East
189   Lansing, MI, USA). Mean values of all data for all parameters were subjected to
190   analysis of variance (ANOVA) by using SAS (version 9) computer software programs
191   [SAS inst., 2002]. A least significant difference (LSD) was used to compare significant
192   means at 5% probability level.

193

194   ## 3.   RESULTS

195   *3.1. Survival rates of Di, Tri, and tetraploid watermelon*

196   Plant survival rates were recorded for all the grafting combinations among diploid (Di),

197   triploid (Tri), and tetraploid (Tetra), grafted by different parts from mother plants on

198   interspecific rootstock during two consecutive seasons, March and August 2018. The

199   statistical analysis showed highly significant differences between Di, Tri, and Tetra

200   when 1N was used as a scion, and the combined analysis for two seasons gave 45.45,

201   55.45, and 78.03% in Di, Tri, and Tetra watermelon respectively. Grafting by AM gave

202   the highest survival rate 93.3, 96.67, and 96.67 % in the first season and 96.67, 100,

203   and 100 % in the second season in Di, Tri, and Tetra watermelon, respectively, but no

204   significant differences were observed between polyploids. Also, branch with 2 nodes

205   (2N) did not show any significant differences between polyploids in terms of survival

206   rate 86.67, 83.33 and, 83.33 % in the first season and 90, 93.33, and 96.67 % in the

207   second season in Di, Tri, and Tetra watermelon respectively, (Figure: 2 and 3) (Figure

208   S2, and S3). Our results also showed the suitability of vegetative propagation using

209   AM, 2N, and 1N branches as scions in tetraploid watermelon, it is a new method for

210   seedless watermelon seed producers.

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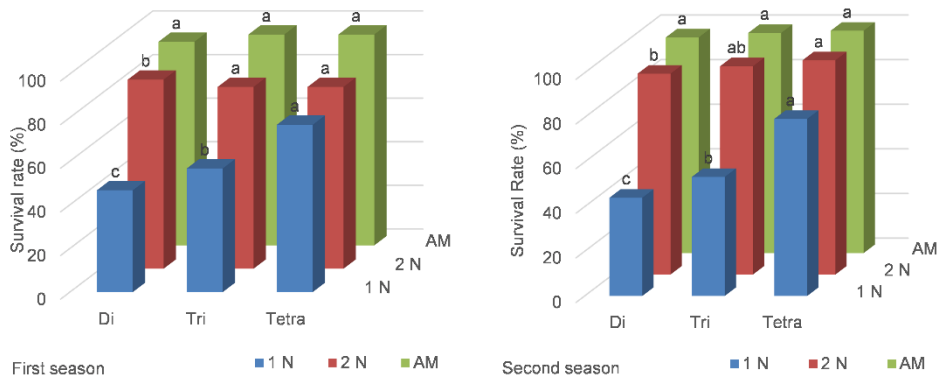
213   ***Figure 2***. *The effect of Scion/rootstocks combinations on the survival rate of polyploid watermelon grafting union at*

214   *15 days after grafting observed a significant difference between AM and 1N.* ***AM****: Apical Meristem,* ***1N****: Branch with*

215   *1Node,* ***2N****: Branch with 2Nodes.*

216

***Figure 3**. Effect of Scion/rootstocks combinations on the survival of polyploid watermelon grafting union at 15 days after grafting. No significant differences between polyploids when AM and 2N were used as scions, but 1N showed highly significant between di and tetraploid. New branches can be observed in cycles. (**A**): Apical Meristem (AM), (**B**) Branch with 2Node (**2N**), (**C**): Branch with 1Nodes (**1N**), **DAG**: Days after Grafting.*

3.2. Measurement of IAA and ZR in the grafting union among Di, Tri, and tetraploid watermelon at different days after grafting

Ploidy level (Di, Tri, and Tetra) exerted a significant effect on IAA content in the graft union at all stages 0, 3, and 15 days after grafting (DAG) (Figure 4). At 0 DAG, the contents of IAA in Tetra were 2.26 and 2.35-fold higher than Di in the first and second season, respectively, while in Tri, IAA was 1.4 and 1.38-fold higher than Di in the first and second season, respectively. Also, the increment rate of IAA in the graft union at 3 DAG in all polyploids; were 23.88, 37.93, and 23.68% in the first season and 26.35, 35.82, and 25.34% in the second season in Di, Tri, and Tetra watermelon, respectively. The content of IAA was higher in Tetra, followed by Tri and Di watermelon at 0, 3, and 15 DAG, respectively.

Whereas ZR showed no significant differences between Di and Tetra at 0 DAG, while at 3 and 15 DAG, there were highly significant differences in both seasons (Figure 4). The results at 3 and 15 DAG showed that the content of the ZR starts to increase significantly in Tetra than Di and Tri. At 3 DAG, the content of ZR was increased by 59.43 and 42.9% during the first, and second season respectively, and by 18.69 and 37% at 15 DAG during both seasons.

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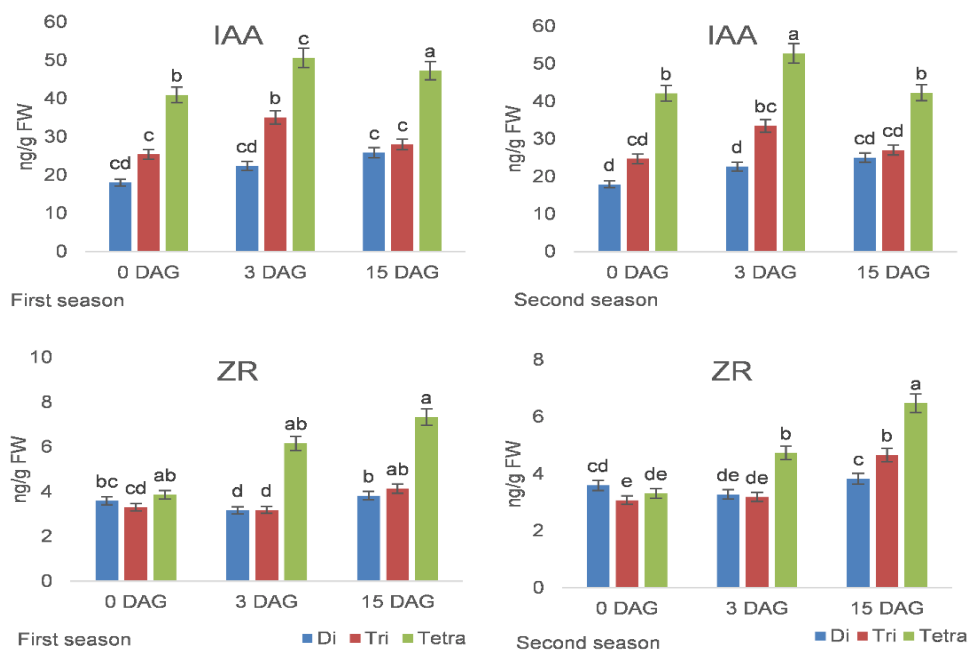
Figure 4. *Effect of Scion/rootstocks combinations on IAA and ZR hormones on polyploids watermelon grafting union at 0, 3, and 15 days during March 2018 and August 2018 cropping seasons. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, and e) in rows indicate significant differences at P < 0.01 using Duncan's multiple range test. **IAA**: Indole acetic acid, **ZR**: Zeatin Riboside, **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.*

3.3 Measurement of Antioxidants (POD, SOD, and CAT) and H_2O_2 in the grafting union among Di, Tri, and tetraploid watermelon at different days after grafting

The increase of antioxidants (AOX) activity such as peroxidase (POD) and catalase (CAT) can scavenge reactive oxygen species (ROS) in plants, thus improving the resistance of plants to stress. Antioxidant enzyme activities were substantially affected by the grafting process between polyploid watermelon [48,49]. After grafting, the results of POD showed highly significant differences between different ploidy watermelons during both seasons (Figure 5). The highest POD activities 2.87 and 2.05-fold was recorded in Tetra at 0 DAG in comparison to Di, and 1.48 and 1.19-fold as compared to Tri in the first and second season, respectively. Higher POD activities in the graft union were observed at 3 DAG in all polyploids, with increment rates of 124.84, 98.88, and 89.83% in the first season and 22.34, 16.25, and 63.21% during the second season in Di, Tri, and Tetra watermelon respectively. While at 15 DAG, the increment rates were (46.86, 47.04, and 47.09%) in the first season and 80.53, 18.64, and 17.74% during the second season in Di, Tri, and Tetra watermelon, respectively.

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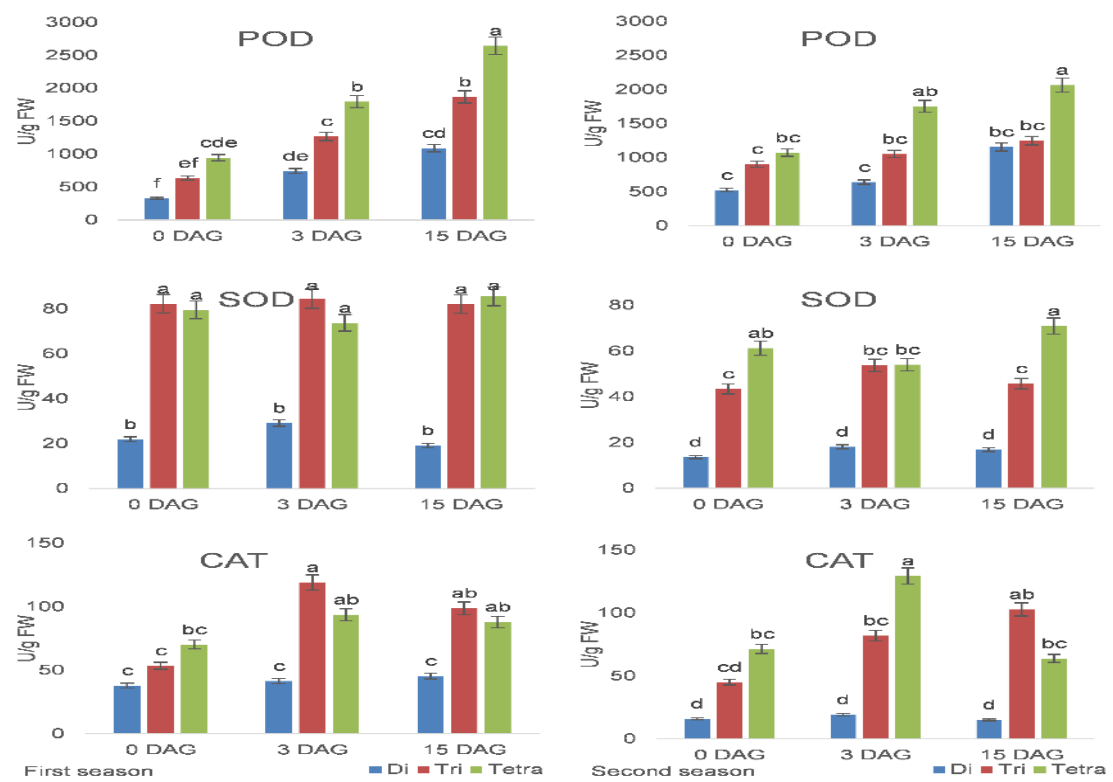
*Figure 5. Effect of Scion/rootstocks combinations on Peroxidase (POD), Superoxide dismutase (SOD), and Catalase (CAT) activities at polyploid watermelons at 0, 3, and 15 days during crop season March 2018 and August 2018 cropping seasons. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, e, and f) in rows indicate significant differences at P < 0.01 using Duncan's multiple range test. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.*

SOD activity was observed higher in Tetra and Tri watermelon as compared to Di watermelon (Figure 5). At 0 DAG, SOD activities were 3.76 and 3.19-fold higher in Tri than diploid in both seasons, respectively. At 3 DAG, SOD activities were 2.9 and 2.97-fold higher than Di in the first and second season, respectively. In comparison, at 15 DAG, the SOD activities in Tri were 4.32 and 2.7-fold higher than Di in both seasons. In Tetra SOD activities at 0 DAG were 3.64 and 4.49-fold higher than Di in the first and second season, respectively, while at 3 DAG, it was 2.53 and 3-fold higher than Di in the first and second season, respectively. Moreover, at 15 DAG, the contents of SOD in Tri were 4.47 and 4.19-fold higher than Di in the first and second season, respectively. Generally, there are significant differences between Tetra and Di at 0, 3, and 15 DAG.

Significantly higher CAT content was observed in Tetra than Di and Tri (Figure 5). At 0 DAG it was 1.85 and 2.35-fold higher in Tetra than Di in the first and second season respectively, and 1.31 and 1.7-fold higher than Tri in the first and second season,

279   respectively. At 0 DAG, CAT activity was 1.4 and 1.38-fold higher than Di in the first

280   and second season, respectively. CAT activities in the graft union start to increase at 3

281   DAG in all polyploids, but the increase at Tri was more than Di and Tetra, with

282   increment rates of 9.19, 122.29, and 33.47% in the first season and 26.35, 35.52, and

283   25.34% in the second season in Di, Tri, and Tetra watermelon respectively.

284   Reactive Oxygen Species (ROS) like hydrogen peroxide (H_2O_2) accumulation leads to

285   cell death, and oxidative damage depends on the balance between the production of

286   AOX and ROS [46,47]. H_2O_2 contents were higher in Triploid than Di and Tetra at 0

287   DAG stage (Figure 6). It was 1.49 and 1.65-fold higher than Di in the first and second

288   season, respectively, and 1.14 and 1.2-fold higher than Tetra in the first and second

289   season, respectively. But the increment rates of H_2O_2 activities in the graft union at

290   Tetra starts to increase at 3 DAG less than Di, and, Tri; the increment rates were 51.51,

291   5.43, and 4.08% in the first season and 42.53, 8.62, and 8.79% in Di, Tri, and Tetra

292   plants respectively. These may be because of the high activities of AOX in Tetra than

293   Di and Tri during the grafting process.

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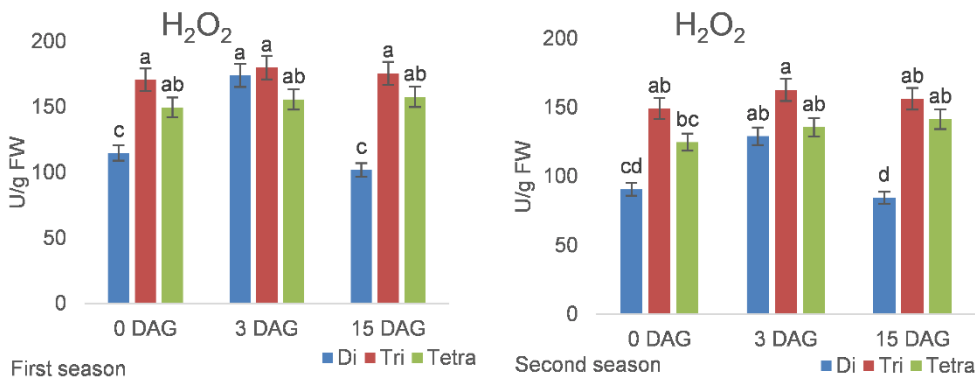
295   ***Figure 6****. Effect of Scion/rootstocks combinations on Hydrogen peroxide (H_2O_2) contents at polyploid watermelon*

296   *grafting union at 0, 3, and 15 days during crop season March and August 2018. The values are means of fifteen*

297   *independent plants (n = 15). Different letters (a, b, c, and d) in rows indicate significant differences at P < 0.01*

298   *using Duncan'ıs multiple range test. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.*

299   *3.4. Measurement of Lignin, phenols, and starch contents in the grafting union among*
300   *Di, Tri, and tetraploid watermelon at different days after grafting*

301   Lignin content was significantly different between Di and Tetra watermelons in both
302   seasons (Figure 7). Lignin contents were higher in Tri, and Tetra than Di at 0 DAG,
303   where it at Tetra were 2.7 and 2.41-fold higher than Di in the first and second season
304   respectively, and at Tri were 2.67 and 2.41-fold higher than Di in the first and second
305   season, respectively. But because of callus induction (branchime cells) in the graft
306   union at 3 DAG stage [18], the lignin contents start to decrease; nevertheless, the
307   decrement rate in Tetra was less than Di. The increment rates of lignin contents in the
308   graft union at 3 DAG were 51.68, 21.5, and 6.33% in the first season, and 51.68, 16.98,
309   and, 12.32% in the second season at Di, Tri, and, Tetra watermelon respectively. At 15
310   DAG, the lignin contents start to increase by 164.32, 55, 08, and, 59.18% in the first
311   season, and 251.37, 264, and, 188.11% in the second season at Di, Tri, and, Tetra
312   watermelon respectively.

313   The results of phenols showed significant differences between Di and Tetra
314   watermelons in both seasons at 3 DAG (Figure 7). The contents of phenols in Tetra
315   were 1.4, 1.7, and 1.1-fold higher than Di at 0, 3, and 15 DAG, respectively, in the first
316   season, and 1.1, 1.3, and 1.2-fold higher than Di at 0, 3, and 15 DAG respectively, in
317   the second season.

318   The results of starch showed highly significant differences between ploidy watermelons
319   in the first season during the grafting process, while in the second season, no significant
320   differences between ploidy levels (Figure 7). The results showed that, the starch
321   contents in Tri and Tetra highly significant than di, where it at 0 DAG were (1.57 and
322   1.48-fold in Tri and Tetra higher than Di in the first season, while in the second season,
323   no significant differences between polyploidy levels. Also, the contents of starch start
324   to increase at 15 DAG in all polyploids, but the increment rates at Tri and Tetra were
325   more than Di 9.68, 14.09, and 16.58% at the first season in Di, Tri, and Tetra
326   watermelon, respectively.

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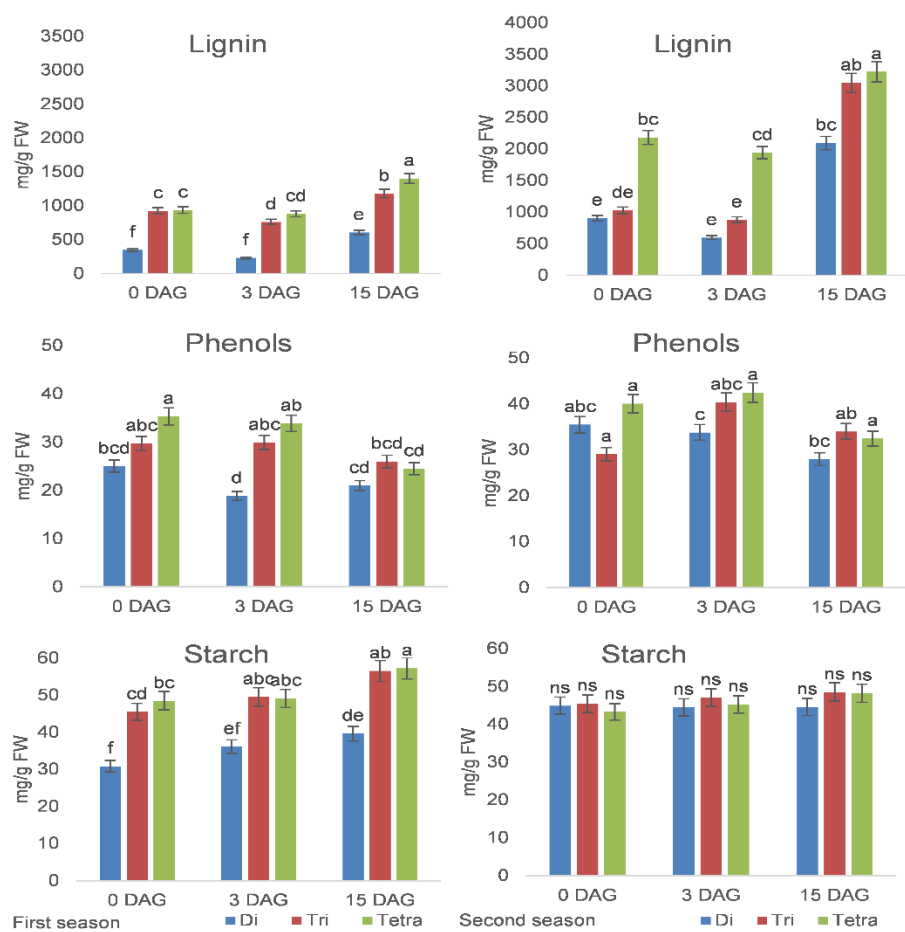
Figure 7. *Effect of Scion/rootstocks combinations on lignin, Phenols, and Starch contents at polyploid watermelon grafting union at 0, 3, and 15 days during crop season March and August 2018. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, e, and f) in rows indicate significant differences at P < 0.01 using Duncan'ıs multiple range test. **Di***: Diploid, **Tri***: triploid, **Tetra***: Tetraploid, **DAG***: Days after grafting, **ns***: not significant.*

3.5 Phylogenetic analysis of selected genes linked to compatibility mechanisms in the grafting union among Di, Tri, and tetraploid watermelon

BlastP searches in the watermelon Genome Database, using *Arabidopsis lyrata* subsp. *Lyrata, Arabidopsis thaliana, Citrullus colocynthis, Citrus sinensis, Coffea canephora, Cucumis melo, Cucumis sativus, Cucurbita maxima, Cucurbita moschata, Cucurbita pepo, Dimocarpus longan, Eutrema salsugineum, Glycyrrhiza uralensis, Gossypium arboretum, Hevea brasiliensis, Kandelia candel, Morus notabilis, Oryza punctate, Oryza sativa, Pinus Sylvestris, Pisum sativum, Populus tremula x Populus tremuloides* (Hybrid aspen)*, Populus trichocarpa, Prunus persica, Ricinus communis, Salvia splendens, Solanum tuberosum, Theobroma cacao, Vitis vinifera, Zantedeschia aethiopica, Zea mays, Ziziphus jujube and Zostera marina* protein sequences for genes linked to H_2O_2, starch, POD, SOD, lignin, phenols, ZR, IAA, and CAT were used as a

345 query, permitted the identification of candidate genes in watermelon. Watermelon

346 genes having high homology are summarized in the (Figure 8) (Table S1).

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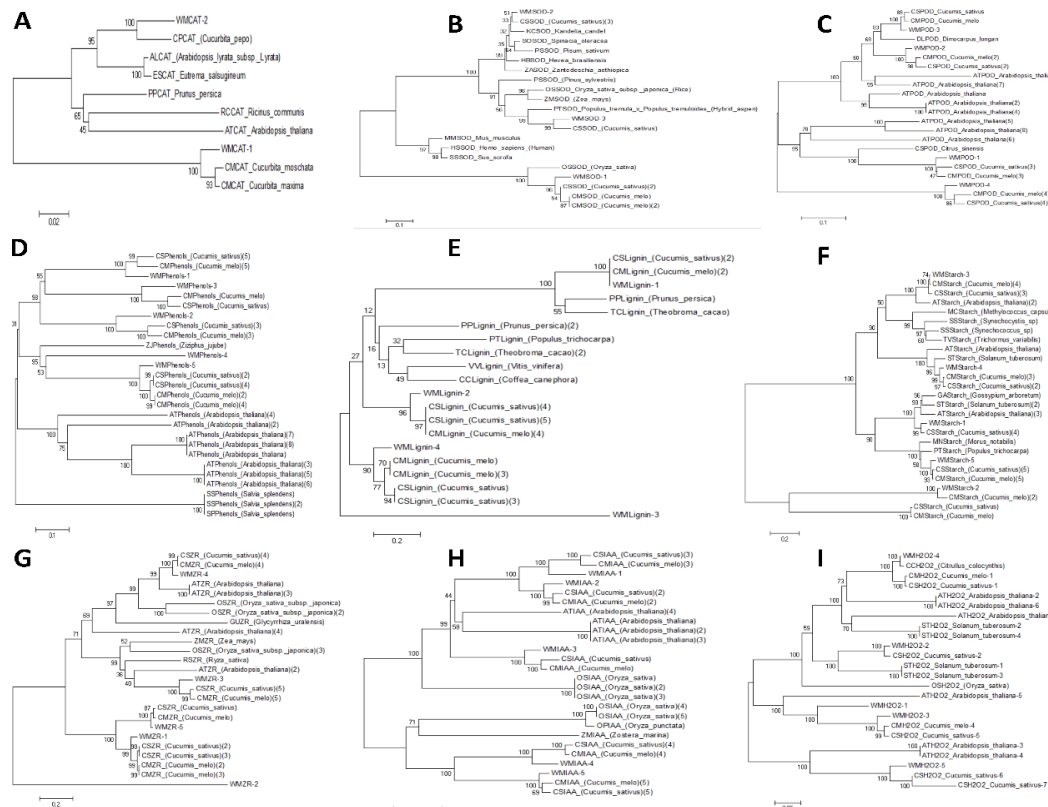

350 **Figure. 8.** Maximum likelihood phylogeny of watermelon genes encoding key enzymes and transporters involved

351 graft compatibility with those from *Arabidopsis lyrata* subsp. *Lyrata, Arabidopsis thaliana, Citrullus colocynthis,*

352 *Citrus sinensis, Coffea canephora, Cucumis melo, Cucumis sativus, Cucurbita maxima, Cucurbita moschata,*

353 *Cucurbita pepo, Dimocarpus longan, Eutrema salsugineum, Glycyrrhiza uralensis, Gossypium arboretum, Hevea*

354 *brasiliensis, Kandelia candel, Morus notabilis, Oryza punctate, Oryza sativa, Pinus Sylvestris, Pisum sativum,*

355 *Populus tremula x Populus tremuloides* (Hybrid aspen), *Populus trichocarpa, Prunus persica, Ricinus communis,*

356 *Salvia splendens, Solanum tuberosum, Theobroma cacao, Vitis vinifera, Zantedeschia aethiopica, Zea mays,*

357 *Ziziphus jujube,* and *Zostera marina.* The phylogenetic tree was constructed from protein sequences using the

358 neighbor-joining method.

359 *3.6 RT-qPCR analysis of genes regulating hormones, biochemical, and antioxidants in*

360 *Di, Tri, and Tetraploid watermelon at different days after grafting*

361 Expressions of genes regulating *CAT, POD, SOD, H_2O_2, IAA, Cytokinin (ZR), phenols,*

362 *lignin, and starch* were checked at three different stages (0, 3, 15 DAG). Primer 3 was

363 used to design primers for RTq-PCR analysis (Table S2). The range of all PCR

364 products was from 80 to 200bp. The specificity of PCR amplification was observed by

365 monitoring the dissociation curves during qPCR using a Roche LightCycler 480 II

366 [58,63]. Transcript levels of these genes were linked to the compatibility or

367 incompatibility as they are involved in regulating important biochemical and

368   antioxidants. *WM-IAA-1 (ClCG05G010400)*, *WM-ZR-1 (ClCG11G005450)*, had higher

369   expression at all the time points in tetra watermelon followed by triploid and diploid

370   watermelon   in   both   seasons.   *WM-POD-1   (ClCG07G008740)***,**   *WM-CAT-1*

371   *(ClCG11G018720), WM-SOD-1 (ClCG04G005370)* had a higher expression in Tetra as

372   compared to Tri and Di watermelon at all days after grafting (Figure 9). *WM-H$_2$O$_2$-1*

373   *(ClCG10G013100)* had a lower expression level in Tetra watermelon than Tri and Di in

374   both the seasons. Genes regulating starch and lignin contents in watermelon, including

375   *WM-Starch-1   (ClCG01G018090),   WM-Lignin-1   (ClCG11G016110),*   had   high

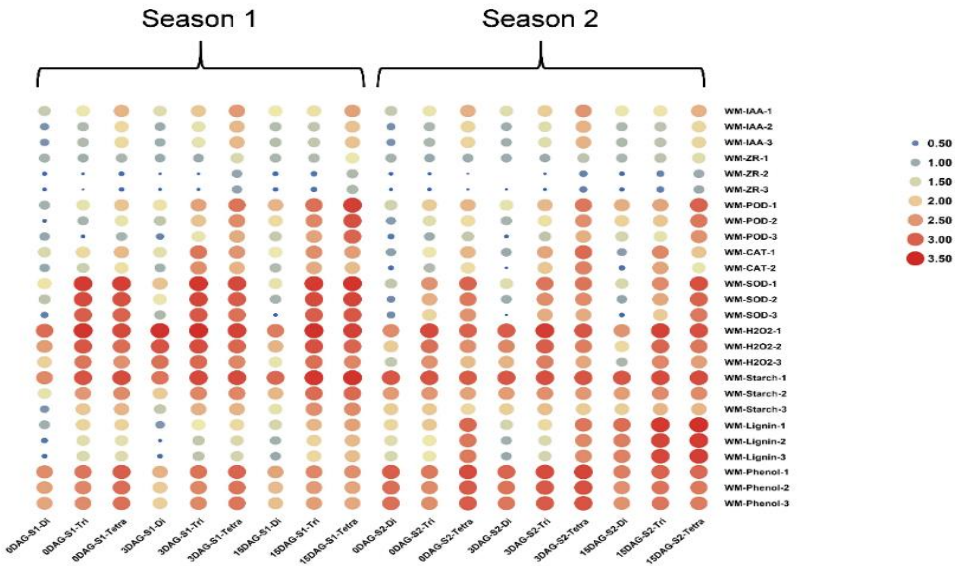
376   expressions in Tetra watermelon as compared to Tri and Di watermelon confirming

377   their active role in starch and lignin accumulation at all the time points of sampling.

378   Among the genes controlling phenolic contents, *WM-Phenol-1 (ClCG09G008770)* had

379   higher expressions in Tri and Di watermelon than Tetra watermelon at all the sampling

380   stages.

381

382   **Figure 9:** *Heatmap of selected genes in Di, Tri, and Tetraploid watermelon at different days after*

383   *grafting in two consecutive seasons.*

384

385   **4. Discussion:**

386   Triploid watermelon seeds are produced by cross-pollination between Di and Tetra, but

387   Tetra seeds production is a very hard process [11]. So it is important to find a more

388   economical way to increase the number and quality of triploid seeds [13]. In this study,

389   we tested three different parts from mother plants as a scion, apical meristem (AM),

390   branch with 1, and 2 nodes (1N and 2N) to increase the number of plants using

391   vegetative propagation from mother plants with grafting technique. Our results showed

392   a higher survival rate (Figure 2) with three kinds of branches used in Tetra plants,

393   whereas in case of triploid AM and 2N give a high survival rate while 1N gave a low

394   survival rate, but in Di just AM gave high survival rate. Suggesting that Tetra grafted

395   with branch with 1 anad 2 nodes and apical meristem, triploid with gragted with AM

396   and 2N and Di grafted with AM are the best possible way of vegetative propagation in

397   watermelon.

398   Plant hormones play important roles in plant growth, development, and response to

399   biotic and abiotic cues and vascular formation in the graft junction [15,32,33,40-45].

400   Auxin, and cytokinin, play an important role in regulating stock–scion interactions

401   [18,36]. Cell divisions happened within 2-3 DAG in the graft junction and have the

402   highest hormone levels during this period [18,39,40,64]. During the grafting process, the

403   peak of IAA is observed to occur within 3 DAG in the scion, as reported by [65]. IAA

404   and Zeatin Riboside (ZR) are required for vascular bundle regeneration in the graft

405   union [37,38]. The main cause of incompatibility is the occurrence of hormonal

406   imbalance [34]. A low indole-3-acetic acid (IAA) content in incompatible combinations

407   may then affect the differentiation of xylem and phloem, as well as lignification

408   [17,28,66]. We compared the IAA and ZR contents in the graft union between Di, Tri,

409   and Tetra watermelon (Figure 4). The IAA concentration in the Tetra combination was

410   significantly higher than that in the Di, and Tri combination ($p < 0.05$). Our results

411   showed high compatibility in Tetra which has high content and high increment rates of

412   hormones than Di especially at 3 DAG (critical period) and 15 DAG, this can explain

413   high survival rates in Tetra, this results were agreement with [67-69]

414   On the other side, incompatibility results from the stress-induced during the healing

415   response, as reported earlier by [41]. In this study in (Figure 5), POD, SOD, and CAT

416   activities showed a high increase during the healing process in Tetra and Tri. Also, the

417   content of H_2O_2 (Figure 6) didn't increase during the healing process in Tri, because of

418   the high activities of antioxidants, which leads to scavenging oxygen radicals. These
419   results were in accordance with [70-74], who found that genome duplication gave high
420   resistance for salt stress because of the high contents of hormones and highly activities
421   of antioxidants more than Di. Also, ploidy results in chromosome doubling leading to
422   gene doubling, which results in higher expression, thus causing an increase in protein
423   contents [75]. Polyploidy is more tolerant of abiotic stress than Di it was reported in
424   watermelon [76], rice [73,77], citrus [72], black locust [78], honeysuckle [79], kinnow
425   mandarin [80], cotton [81], and rangpur lime [82]. Peroxidase and catalase activities were
426   increased in the grafted plants [83].

427   Generally, antioxidant enzymes were usually studied in the responses to abiotic and
428   biotic stresses, but not often for graft stress; in this study, we suggest grafting as stress
429   because of, cut or wound stress, complete dark, and high humidity stress, especially in
430   the first three days after grafting (healing response) [12,41]. The most important critical
431   period in the grafting healing process was at 2 and 3 days after grafting [18]. The results
432   in both seasons showed that the activities of SOD, POD, and CAT were significantly
433   different at the grafting healing process between polyploid watermelons (Figure 6). It
434   has been reported already by [49,83] that high activities of POD and CAT, during the
435   healing process have a high ability to scavenge reactive oxygen species (ROS) and
436   hydrogen peroxide (H_2O_2) in plants. H_2O_2 product when the plant is wounded or
437   stressed and caused cell death [84].

438   Higher enzymatic activities and accumulation of phenolic compounds are typically
439   associated with plant stress resistance [85]. Phenols contribute to the elimination of
440   reactive oxygen species (ROS) [86]. In our results, the content of phenols was higher in
441   Tetra than Di and Tri in all stages, but because of the accumulation of phenols leads to
442   incompatibility [31,32], a higher decrease in phenol contents were observed at later
443   stages of grafting (15 DAG) (Figure 7). The decrement rates between 3 and 15 DAG in
444   Tetra were (38.24, and 30.77%) in the first and second season, respectively, while in
445   Di, the content of phenols increase at 15 DAG in the first season and decrease (27%) in
446   the second season. With Tri, the decrement rates were (15, and 18.7%) at 15 DAG in
447   the first and second season, respectively. These results can explain the high
448   compatibility of Tetra and low compatibility in a Di. Also, our results are similar to
449   [85], who found that the phenolic content and lignin content were higher in the
450   compatible combination.

451   ## 5. Conclusion

452   Seedless watermelons seeds production faces many problems. The current study
453   concludes that vegetative propagation by branches grafting could be used in Tri and
454   Tetra watermelon seed production. The high contents of hormones and high activities
455   of antioxidants in Tetra more than Di because of the genome duplication, which gave
456   high compatibility and high survival rates of grafting. Grafting using branches can be a
457   good alternative to promote vegetative propagation in Tetra watermelon. These
458   findings add significant information to our existing knowledge of compatibility in
459   watermelon by ultimately helping in vegetative propagation, breeding watermelon
460   varieties of desirable values.

461

462 **Conflict of interest**

463 The authors declare that the research was conducted in the absence of any commercial

464 or financial relationships that could be construed as a potential conflict of interest.

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475 **Supplementary Materials:**

476 **Figure S1:** Splice grafting method steps in polyploid watermelon plants

477 **Figure S2:** Effect of Scion/rootstocks combinations on the survival of polyploid

478 watermelon grafting using (AM) as scion. (A): at 0 DAG, (B) 15 DAG, and (C): 25

479 DAG, DAG: Days after Grafting.

480 **Figure S3**: Effect of Scion/rootstocks combinations on the survival of polyploid

481 watermelon grafting using (2N) as scion. (A): at 15 DAG, and (B) 25 DAG.

482 **Figure S4:** Structure of genes involved in graft compatibility during watermelon

483 grafting process. Yellow boxes and black lines indicating exons and introns

484 respectively, while blue boxes at the both end of each gene indicates untranslated

485 regions (UTRs).

486 **Table S1:** Genes linked to graft compatibility having high homology with other genes

487 selected from different plant species.

488 **Table S2:** Primers used for qRT-PCR in this study.

489 **Author Contributions**

490 KMO and WL conceived and designed the experiments. KMO performed the

491 experiments. KMO and MJU analyzed the data and wrote the manuscript. HG, EM,

492 WD, PY, HZ, SZ, XL, NH, CG, MA, and EL participated in the experimentations and

493   analysis. MJU, MA, HZ, and WL edited the manuscript. All authors read and approved

494   the last version.

495

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503   **Data availability**

504   The authors declare that the data supporting the study findings are presented in the

505   article, and Supplementary Information files are available from the corresponding

506   author upon request.

507

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