Long-Lasting Graft Union Compatibility Enigma explained by Comparative physiological and biochemical mechanisms in Di, Tri, and Tetraploid Watermelon (*Citrullus lanatus* L.) grafted by Branches

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Abstract

Polyploid seed production is complicated, and costly work, produce a fewer number of seeds/fruits as compared to diploid seeds. Here we investigated the interactive effect of a new grafting technique of polyploid watermelon scion onto rootstock on plants' survival rate. In this study, three different branches, apical meristem (AM), branch with 1 node (1N), and 2 nodes (2N) from di, tri, and tetraploid watermelon plants, were used as scion and grafted onto squash rootstock. The results showed highly significant differences between polypoid watermelon when 1N was used as a scion. Tetraploid showed maximum survival rates, higher contents of hormones, carbohydrates, and antioxidants activities, compared to diploid. Here, we also performed applied sucrose exogenously, on the rootstocks seedlings before grafting to enhance survival rates. Significant survival rates were observed in the case of 2% sucrose application in all polyploids when 1N was used as a scion. RTq-PCR results confirm that the expression of genes linked to compatibility is consistent with the carbohydrates, hormonal and antioxidants activities. This study provides an alternative and economical approach to produce more tetraploid and triploid plants for breeding and seed production by using branches as scions, furthermore, provides more understanding of graft compatibility.

Keywords: watermelon, polyploidy, grafting, branches, compatibility

1. Introduction

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].

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 [14]. All of these steps can determine the future of a grafted plant [15]. However, still now no general rules, on how these treatments affect incompatibility responses [16,17]. To date, the cause and underlying mechanism for grafting incompatibility remain elusive, and there is no report on genes associated with grafting incompatibility [18,19]. More research work needs to be done to fully understand the mechanisms of graft compatibility and incompatibility [20,21].

In fruit trees, graft incompatibility occurs due to the accumulation of phenolic compounds [22]. [23] found that reduced auxin and lignification was associated with high phenols in incompatible grafts and may suggest weak graft unions, or reactive oxygen species (ROS) [24-27], or hormone imbalance [16,28,29]. IAA, and cytokinin, play an important role in regulating stock–scion interactions [30], and required for vascular bundle regeneration in the graft union [31,32]. Plant hormones are important for vascular formation; auxin is important for the differentiation of vascular tissues and wound healing [20,26,27,33-38]. Reactive Oxygen Species (ROS) accumulation leads to cell death, and oxidative damage depends on the balance between the production of

antioxidant AOX enzymes and ROS [39,40]. Increase the activity of defense enzymes such as Peroxidase POD, and catalase CAT can scavenge ROS in plants, and improving the resistance of plants to stress [41,42]. POX and CAT convert H_2O_2 to H_2O [43]. So the difference relation between AOX and ROS during the healing process could be used as a rapid mechanism to verify incompatibility [44]. Lignin is abundant in woody plants and primarily contributes strength to the cell wall; so, decreased lignin would be antagonistic to a strong graft union [21].

Furthermore, carbohydrates play an important role as a source of energy for plant cellular activities [45,46]. The development of the graft union depends on the content of carbohydrates in the rootstock and the scion at the time of grafting, high levels of carbohydrates, as well as auxin and cytokinin, are required for successful callus formation, and there can be an interaction between rootstock and scion cultivars in carbohydrate utilization, which can affect the level of starch depletion that occurs during callus development, the survival rate was positively correlated with an increase in starch content [46,47].

Previous researches with perennial crops suggest that increasing carbohydrate concentrations in grafted tissues may increase grafting success, for example with grapes there is a positive relationship between carbohydrate level and callus formation in grafted grape which led to an increase in the survival rate of grafted transplants [48]. Likewise, grape studies, macadamia, litchi, avocado, and watermelon seedlings suggested that an increase in starch content of the scion is positively correlated with an increase in grafting success, [46-50].

The purpose of this research was to study the effect of genome duplication on graft compatibility, by comparing the factors and parameters which lead to graft compatibility or incompatibility. This study provides information at the molecular and physiological levels for the mechanism compatibility in polyploid watermelons, which should explain the mechanism of compatibility in polyploid watermelon, also to try a new technique for vegetable propagation by using branches as scions, three different branches were taken from mother plants and graft on squash rootstock to study the success of this method, and how to increase the survival rate by sucrose application. This method will add an option for breeders and seed producers to save time and money using an asexual method to increase plant numbers.

2. RESULTS

2.1. Survival rates of Di, Tri, and tetraploid watermelon

Plant survival rates were recorded for all the grafting combinations among diploid (Di), triploid (Tri), and tetraploid (Tetra), grafted by different parts from mother plants on interspecific rootstock during two consecutive seasons, March and August 2019. The statistical analysis showed highly significant differences between Di, Tri, and Tetra when 1N was used as a scion, and the combined analysis for two seasons gave 45.45, 55.45, and 78.03% in Di, Tri, and Tetra watermelon respectively. Grafting by AM gave the highest survival rate 93.3, 96.67, and 96.67 % in the first season and 96.67, 100, and 100 % in the second season in Di, Tri, and Tetra watermelon, respectively, but no significant differences were observed between polyploids. Also, branch with 2 nodes (2N) did not show any significant differences between polyploids in terms of survival rate 86.67, 83.33 and, 83.33 % in the first season and 90, 93.33, and 96.67 % in the second season in Di, Tri, and Tetra watermelon respectively, (Figure: 1 and 2) and the success of grafting after 30 DAG (Figure S1, and S2). Our results also showed the suitability of vegetative propagation using AM, 2N, and 1N branches as scions in tetraploid watermelon, it is a new method for seedless watermelon seed producers.
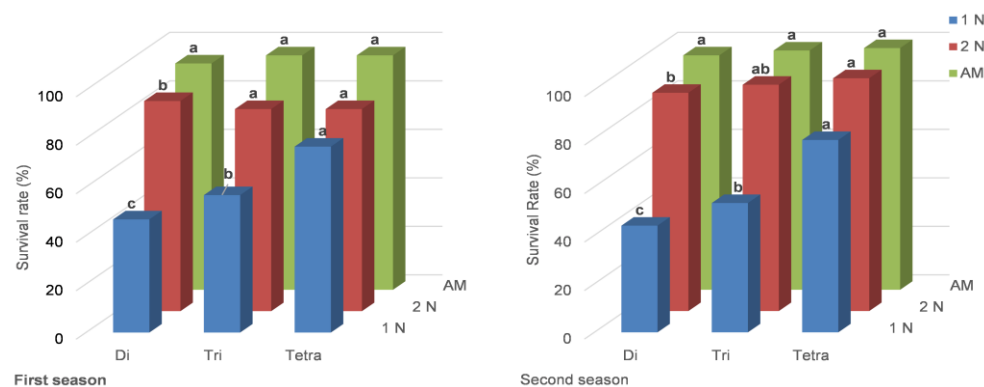

Figure 1. The effect of Scion/rootstocks combinations on the survival rate of polyploid watermelon grafting union at 15 days after grafting observed a significant difference between AM and 1N. **AM**: Apical Meristem, **1N**: Branch with 1Node, **2N**: Branch with 2Nodes.

Figure 2. 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 a 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.

2.2. 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 3). 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 3). 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 seasons respectively, and by 18.69 and 37% at 15 DAG during both seasons.
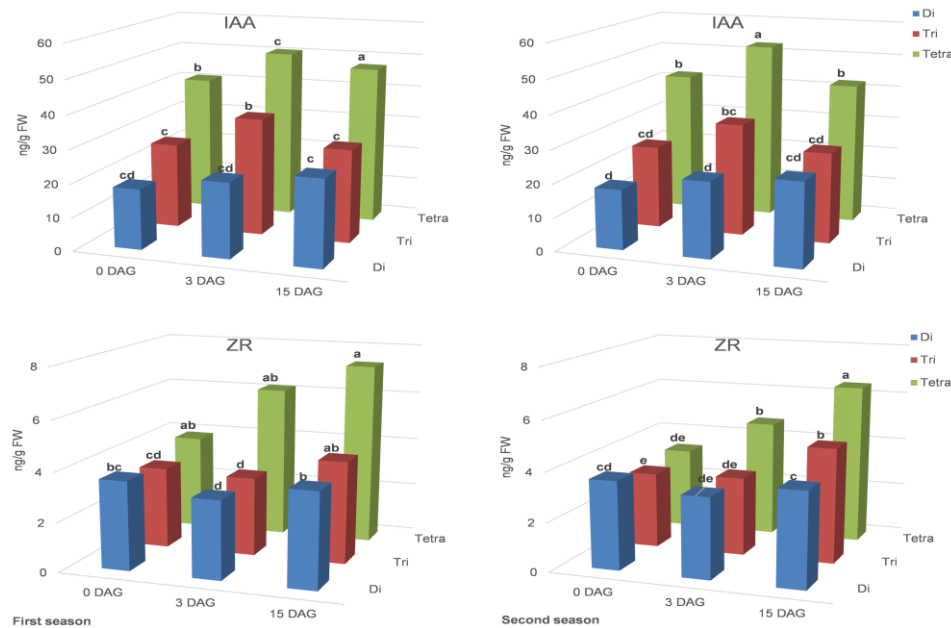
Figure 3. Effect of Scion/rootstocks combinations on IAA and ZR hormones on polyploids watermelon grafting union at 0, 3, and 15 days during March 2019 and August 2019 cropping seasons. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, and e) in rows indicate significant differences at P < 0.01 using Duncan'ıs multiple range test. **IAA**: Indole acetic acid, **ZR**: Zeatin Riboside, **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.

2.3 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 [41,42]. After grafting, the results of POD showed highly significant differences between different ploidy watermelons during both seasons (Figure 4). 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.
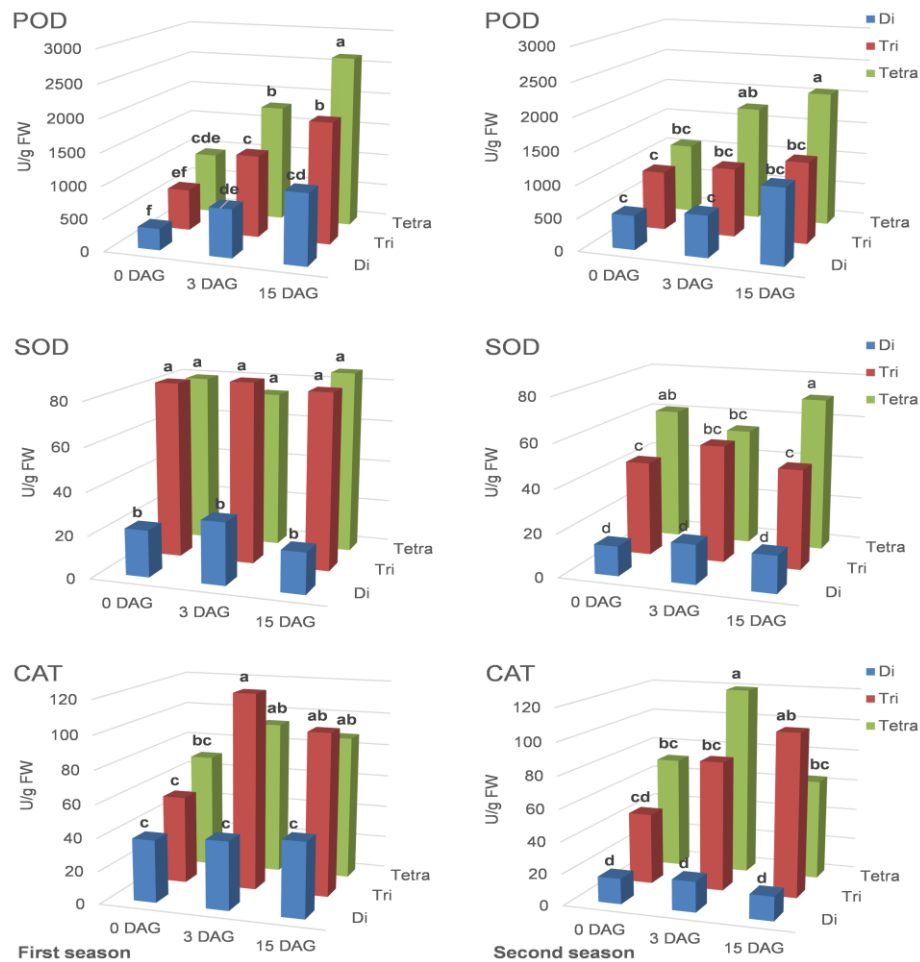
Figure 4. Effect of Scion/rootstocks combinations on Peroxidase (POD), Superoxide dismutase (SOD), and Catalase (CAT) activities at polyploid watermelons at 0, 3, and 15 days during crop season March 2019 and August 2019 cropping seasons. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, e, and f) in rows indicate significant differences at P < 0.01 using Duncan'ıs 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 4). 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 seasons, 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 4). 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, respectively. At 0 DAG, CAT activity was 1.4 and 1.38-fold higher than Di in the first and second seasons, respectively. CAT activities in the graft union start to increase at 3 DAG in all polyploids, but the increase at Tri was more than Di and Tetra, with increment rates of 9.19, 122.29, and 33.47% in the first season and 26.35, 35.52, and 25.34% in the second season in Di, Tri, and Tetra watermelon respectively.

Reactive Oxygen Species (ROS) like hydrogen peroxide (H_2O_2) accumulation leads to cell death, and oxidative damage depends on the balance between the production of AOX and ROS [39,40]. H_2O_2 contents were higher in Triploid than Di and Tetra at 0 DAG stage (Figure 5). It was 1.49 and 1.65-fold higher than Di in the first and second season, respectively, and 1.14 and 1.2-fold higher than Tetra in the first and second season, respectively. But the increment rates of H_2O_2 activities in the graft union at Tetra starts to increase at 3 DAG less than Di, and, Tri; the increment rates were 51.51, 5.43, and 4.08% in the first season and 42.53, 8.62, and 8.79% in Di, Tri, and Tetra plants respectively. These may be because of the high activities of AOX in Tetra than Di and Tri during the grafting process.
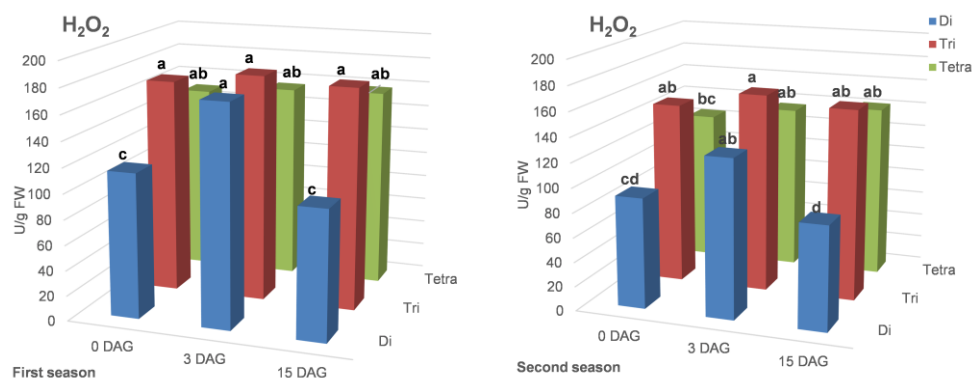

Figure 5. Effect of Scion/rootstocks combinations on Hydrogen peroxide (H_2O_2) contents at polyploid watermelon grafting union at 0, 3, and 15 days during crop season March and August 2019. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, and d) in rows indicate significant differences at P < 0.01 using Duncan'ıs multiple range test. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.

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

Lignin content was significantly different between Di and Tetra watermelons in both seasons (Figure 6). Lignin contents were higher in Tri, and Tetra than Di at 0 DAG, where it at Tetra were 2.7 and 2.41-fold higher than Di in the first and second season respectively, and at Tri were 2.67 and 2.41-fold higher than Di in the first and second season, respectively. But because of callus induction (branchime cells) in the graft union at 3 DAG stage [51], the lignin contents start to decrease; nevertheless, the decrement rate in Tetra was less than Di. The increment rates of lignin contents in the graft union at 3 DAG were 51.68, 21.5, and 6.33% in the first season, and 51.68, 16.98, and, 12.32% in the second season at Di, Tri, and, Tetra watermelon respectively. At 15 DAG, the lignin contents start to increase by 164.32, 55, 08, and, 59.18% in the first season, and 251.37, 264, and, 188.11% in the second season at Di, Tri, and, Tetra watermelon respectively.

The results of phenols showed significant differences between Di and Tetra watermelons in both seasons at 3 DAG (Figure 6). The contents of phenols in Tetra were 1.4, 1.7, and 1.1-fold higher than Di at 0, 3, and 15 DAG, respectively, in the first season, and 1.1, 1.3, and 1.2-fold higher than Di at 0, 3, and 15 DAG respectively, in the second season.

The results of starch showed highly significant differences between ploidy watermelons in the first season during the grafting process, while in the second season, no significant differences between ploidy levels (Figure 6). The results showed that, the starch contents in Tri and Tetra highly significant than di, where it at 0 DAG were (1.57 and 1.48-fold in Tri and Tetra higher than Di in the first season, while in the second season, no significant differences between polyploidy levels. Also, the contents of starch start to increase at 15 DAG in all polyploids, but the increment rates at Tri and Tetra were more than Di 9.68, 14.09, and 16.58% at the first season in Di, Tri, and Tetra watermelon, respectively.
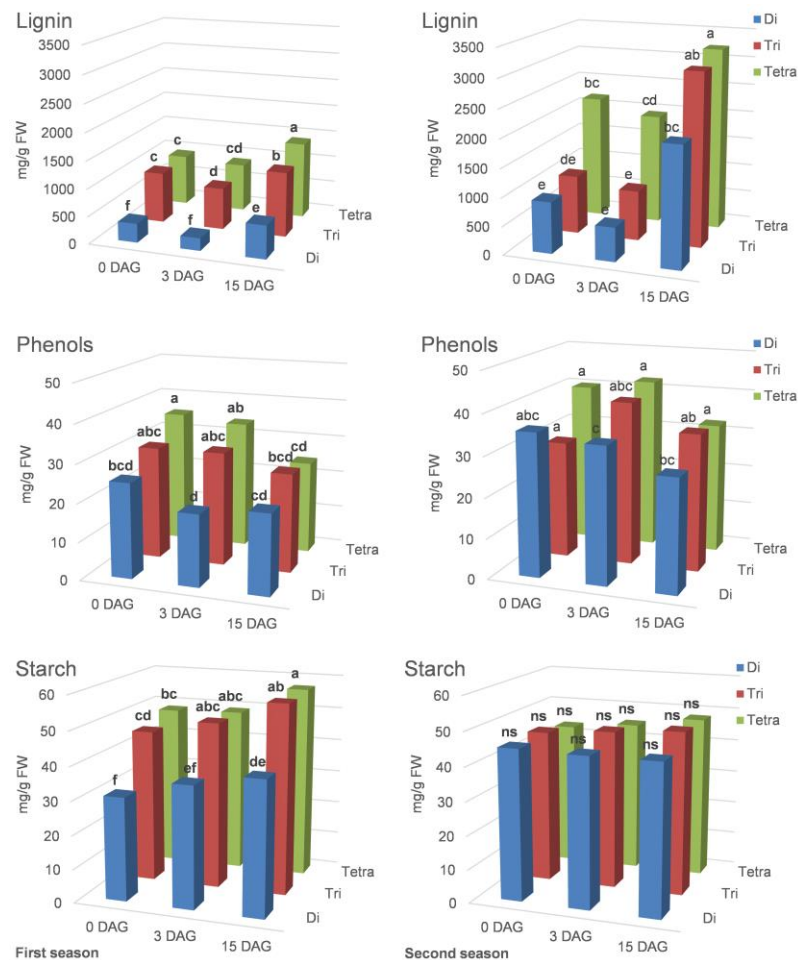
Figure 6. Effect of Scion/rootstocks combinations on lignin, Phenols, and Starch contents at polyploid watermelon grafting union at 0, 3, and 15 days during crop season March and August 2019. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, e, and f) in rows 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.

Heat map (Figure S4A and S4B) first and second season visualization of all hormones and biochemical, showed a widespread change in concentrations in the grafting union at different stages in polyploid watermelon. Notably, the great majority of compounds showed grafting stage-related changes in quantity, either increasing or decreasing, in different polyploid.

2.5 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 query, permitted the identification of candidate genes in watermelon. Watermelon genes having high homology are summarized in (Figure 7) (Table S1).
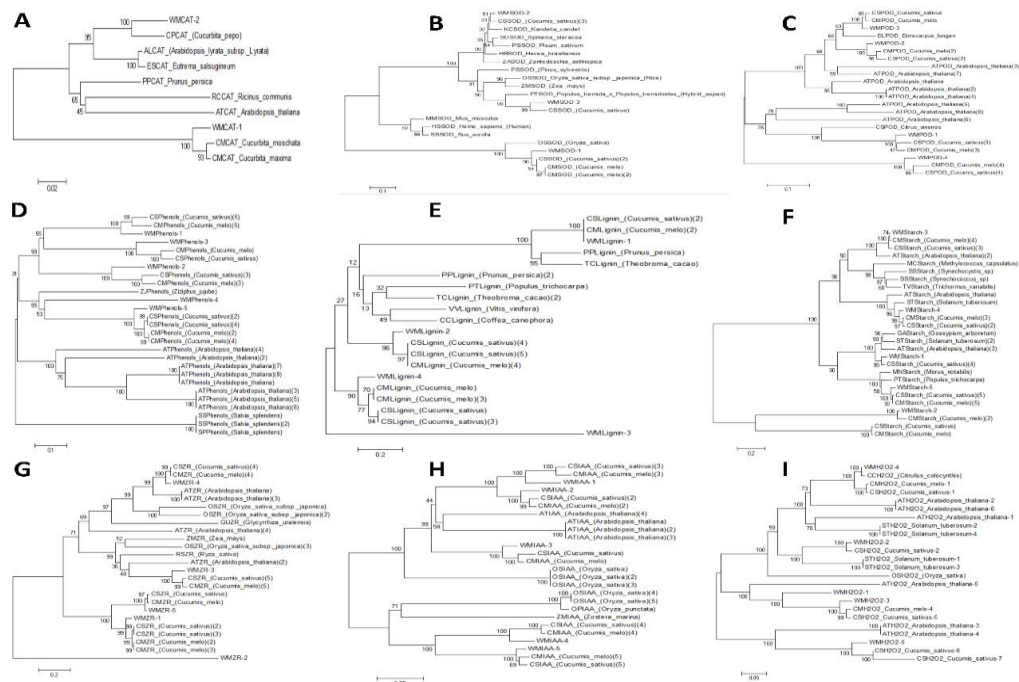

Figure. 7. Maximum likelihood phylogeny of watermelon genes encoding key enzymes and transporters involved graft compatibility with those from *Arabidopsis lyrata* subsp. *Lyrata, Arabidopsis thaliana, Citrullus colocynthis, Citrus sinensis, Coffea canephora, Cucumis melo, Cucumis sativus, Cucurbita 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*. The phylogenetic tree was constructed from protein sequences using the neighbor-joining method.

2.6. RT-qPCR analysis of genes regulating hormones, biochemical, and antioxidants in Di, Tri, and Tetraploid watermelon at different days after grafting

Expressions of genes regulating *CAT, POD, SOD, H_2O_2, IAA, Cytokinin (ZR), phenols, lignin, and starch* were checked at three different stages (0, 3, 15 DAG). Primer 3 was used to design primers for RTq-PCR analysis (Table S2). The range of all PCR products was from 80 to 200bp. The specificity of PCR amplification was observed by

monitoring the dissociation curves during qPCR using a Roche LightCycler 480 II [52,53]. Transcript levels of these genes were linked to the compatibility or incompatibility as they are involved in regulating important biochemical and antioxidants. *WM-IAA-1 (ClCG05G010400)*, *WM-ZR-1 (ClCG11G005450)*, had higher expression at all the time points in tetra watermelon followed by triploid and diploid watermelon in both seasons. *WM-POD-1 (ClCG07G008740)*, *WM-CAT-1 (ClCG11G018720), WM-SOD-1 (ClCG04G005370)* had a higher expression in Tetra as compared to Tri and Di watermelon at all days after grafting (Figure 8). *WM-H$_2$O$_2$-1 (ClCG10G013100)* had a lower expression level in Tetra watermelon than Tri and Di in both the seasons. Genes regulating starch and lignin contents in watermelon, including *WM-Starch-1 (ClCG01G018090)*, *WM-Lignin-1 (ClCG11G016110),* had high expressions in Tetra watermelon as compared to Tri and Di watermelon confirming their active role in starch and lignin accumulation at all the time points of sampling. Among the genes controlling phenolic contents, *WM-Phenol-1 (ClCG09G008770)* had higher expressions in Tri and Di watermelon than Tetra watermelon at all the sampling stages.
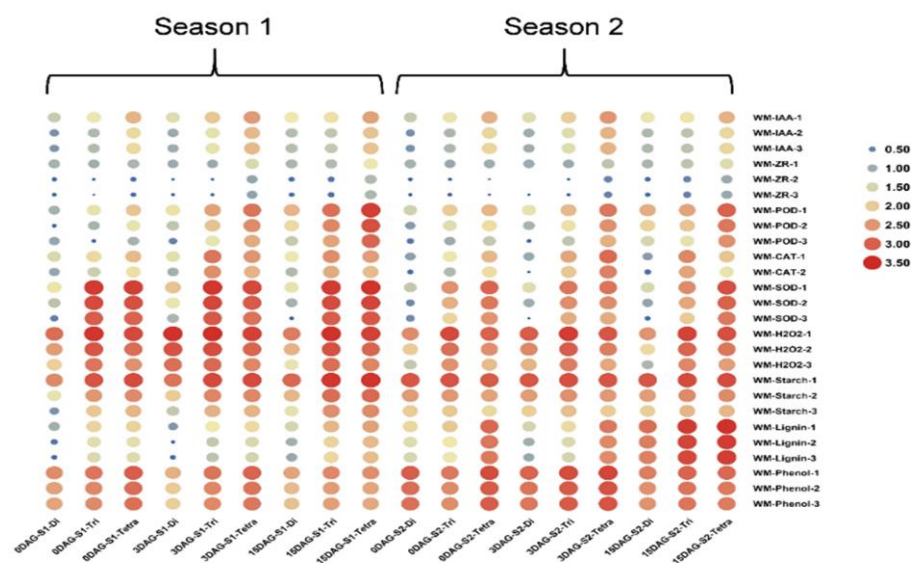

Figure 8: Heatmap of selected genes in Di, Tri, and Tetraploid watermelon at different days after grafting in two consecutive seasons.

2.7. Survival rates of Di, Tri, and Tetraploid watermelon after sucrose drench with different concentrations on rootstocks seedlings

The highest survival rates were recorded with 2% sucrose application i.e. 74.23, 83.13, and 92.57 % in the first season and 74.24, 83.13, and 94.57 % in the second season in Di,

Tri, and Tetra watermelon, respectively. Whereas the survival rates were 62.33, 86.6, and 89.07 % in the first season and 62.33, 78.6, and 87.07 % in the second season in Di, Tri, and Tetra watermelon, respectively were observed in the case of 3% sucrose application. Significant differences between polyploids were observed after sucrose application (Figure 9). These results indicate that drench applications of 2% sucrose solution to rootstocks seedlings before grafting can increase survival rate when grafted by the branch with 1N.
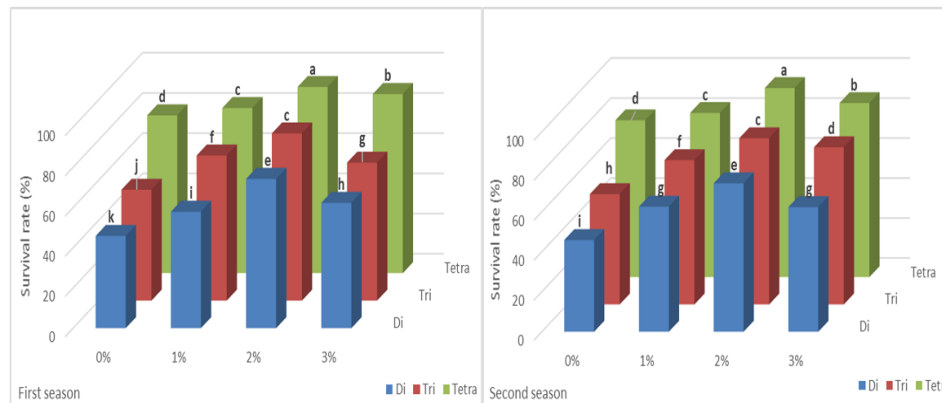

Figure 9. The effect of sucrose [1%, 2%, 3% sucrose, and water (control)] application on Scion/rootstocks combinations grafted by 1N on the survival rate of polyploid watermelon grafting union at 15 DAG. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid.

2.8. Measurement of starch contents in the grafting union among Di, Tri, and Tetraploid watermelon after sucrose application with different concentrations at different grafting development stages.

An increase in starch accumulation was observed with an increase in the concentration of sucrose application. Starch accumulation in grafting union in polyploid watermelon at 3 and 15 DAG was the highest for plants that received a 3% sucrose solution, followed by plants that received 2% sucrose solution (Figure 10). Compared to control, 3% sucrose application gave the highest starch accumulations. During the first season, starch contents of 131, 125.45, and 142.27 mg/g FW, were in case of diploid, 80.58, 85.19, and 76 mg/g FW in case of triploid and 196.35, 223.27, and 232.24 mg/g FW in case of tetraploid at 0, 3, and 15 DAG respectively. While in the second starch contents of 61.69, 105.86, and 116.9 mg/g FW were in case of diploid, 79.23, 88.26, and 105.09 mg/g FW in case of triploid, and 196.35, 223.27, and 232.24 mg/g FW in case of tetraploid at 0, 3, and 15 DAG respectively. On the other hand, In the case of 2% sucrose application, the highest survival rates were observed among all the

polyploids, interestingly, the accumulation of starch in the grafting union at 3 DAG stage (critical period) was significantly higher in Tetra than in the Tri and Diploid, i.e. 2.13 and 1.77-fold higher than Di in the first and second seasons, respectively, and 1.58 and 1.51-fold higher than Tri in the first and second seasons, respectively, These results indicate that sucrose led to an increase in the accumulation of starch in the grafted plants and thus increasing the survival rates in polyploid watermelon.
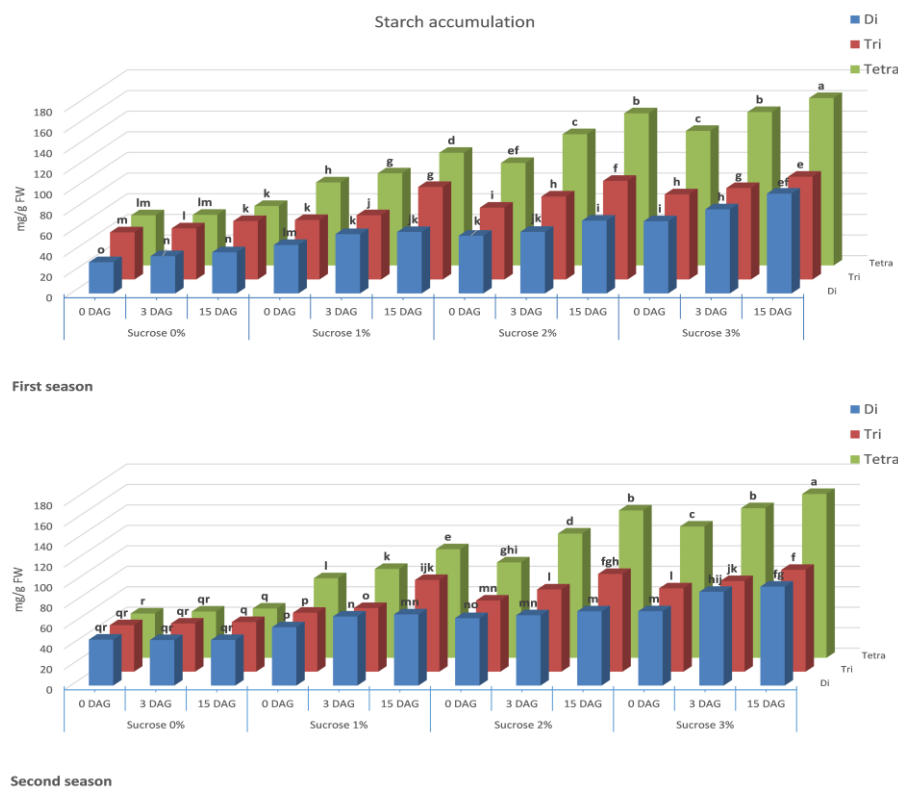

Figure 10. The effect of sucrose application on Scion/rootstocks combinations grafted by 1 N on starch contents on polyploids watermelon grafting union at 0, 3, and 15 days after grafting during March 2019 and August 2019 cropping seasons. The values are means of fifteen independent plants (n = 15). Different letters (a, b, c, d, and e) in rows indicate significant differences at P < 0.01 using Duncan'ıs multiple range test. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.

2.9. Principal component analysis (PCA) in a polyploid watermelon after sucrose treatment with different concentrations (0, 1, 2, and 3%)

Hormones and biochemicals were determined at 0, 3, and 15 DAG, after sucrose application. PCA of the hormones and biochemical contents after sucrose applications showed that IAA and AOXs are sharing the same cluster in both seasons, and starch, lignin, and ZR sharing the same cluster Thus, it can be assumed that they are the key components influencing the compatibility among polyploid watermelon in the grafting union at different days after grafting i.e. 0, 3 and 15 DAG. PCA (Figure 11) (A, B, C, and

D) showed a clear grouping at different stages (0, 3, And 15 DAG) in the polyploid watermelon with control 0%, 1%, 2%, and 3% sucrose application. (E, F, G, and H) showed the clusters with hormones, starch, anti-oxidants, and other biochemicals.
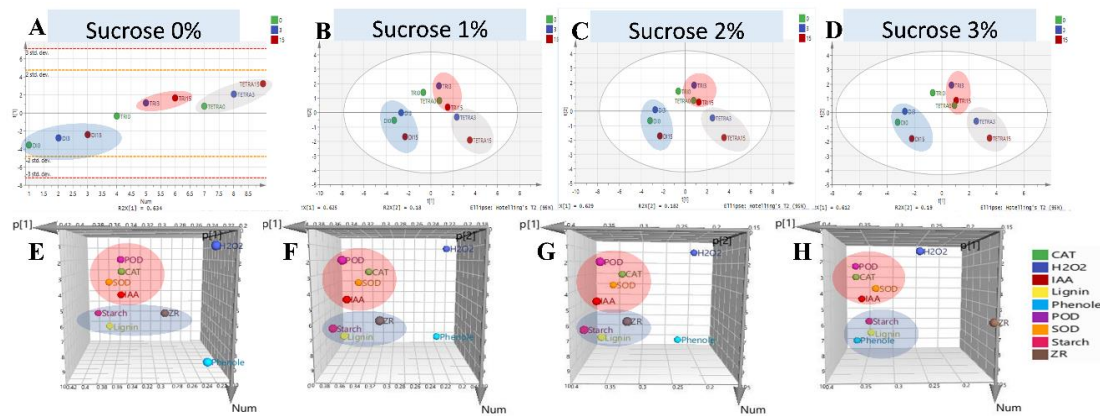

Figure 11. Principal component analysis (PCA) of hormones and biochemical in the grafting union at different stages in polyploid watermelon with different sucrose concentrations (0, 1, 2, 3%). (A, B, C, and D) showed the clusters with polyploids at different grafting stages (0, 3, and 15 DAG). (E, F, G, and H) showed the clusters with hormones, starch, anti-oxidants, and other biochemical. **Di**: Diploid, **Tri**: triploid, **Tetra**: Tetraploid, **DAG**: Days after grafting.

3. Discussion:

Vegatative propagation is useful and had been applied in many other crops i.e. grapes [54], mango [55,56] and peach [57] etc for good survival and to avoid seed production problems. Here we used branch grafting for successful vegetative propagation of different polyploid watermelon. Triploid watermelon seeds are produced by cross-pollination between Di and Tetra, but Tetra seeds production is a very hard process [11]. So, it is important to find a more economical way to increase the number and quality of triploid seeds [13]. In this study, we tested three different parts from mother plants as a scion, apical meristem (AM), branch with 1, and 2 nodes (1N and 2N) to increase the number of plants using vegetative propagation from mother plants with grafting technique. Our results showed a higher survival rate (Figure 2) with three kinds of branches used in Tetra plants, whereas in case of triploid AM and 2N give a high survival rate while 1N gave a low survival rate, but in Di just AM gave high survival rate. Suggesting that Tetra grafted with branch with 1 anad 2 nodes and apical meristem, triploid with gragted with AM and 2N and Di grafted with AM are the best possible ways of vegetative propagation in watermelon.

Plant hormones play important roles in plant growth, development, and response to biotic and abiotic cues and vascular formation in the graft junction [20,26,27,33-38]. Auxin, and cytokinin, play an important role in regulating stock–scion interactions [30,51]. Cell divisions happened within 2-3 DAG in the graft junction and have the highest hormone levels during this period [34,51,58,59]. During the grafting process, the peak of IAA is observed to occur within 3 DAG in the scion, as reported by [60]. IAA and Zeatin Riboside (ZR) are required for vascular bundle regeneration in the graft union [31,32]. The main cause of incompatibility is the occurrence of hormonal imbalance [28]. A low indole-3-acetic acid (IAA) content in incompatible combinations may then affect the differentiation of xylem and phloem, as well as lignification [22,61,62]. We compared the IAA and ZR contents in the graft union between Di, Tri, and Tetra watermelon (Figure 4). The IAA concentration in the Tetra combination was significantly higher than that in the Di, and Tri combination ($p < 0.05$). Our results showed high compatibility in Tetra which has high content and high increment rates of hormones than Di especially at 3 DAG (critical period) and 15 DAG, this can explain high survival rates in Tetra, this results were agreement with [51,63,64]

On the other side, incompatibility results from the stress-induced during the healing response, as reported earlier by [20]. In this study (Figure 5), POD, SOD, and CAT activities showed a high increase during the healing process in Tetra and Tri. Also, the content of H_2O_2 (Figure 6) didn't increase during the healing process in Tri, because of the high activities of antioxidants, which leads to scavenging oxygen radicals. These results were in accordance with [65-68], who found that genome duplication gave high resistance to salt stress because of the high contents of hormones and highly activities of antioxidants more than Di. Also, ploidy results in chromosome doubling leading to gene doubling, which results in higher expression, thus causing an increase in protein contents [68]. Polyploidy is more tolerant of abiotic stress than Di it was reported in watermelon [69], rice [67,68], citrus [66], black locust [70], honeysuckle [71], kinnow mandarin [72], cotton [73], and rangpur lime [74]. Peroxidase and catalase activities were increased in the grafted plants [75].

Generally, antioxidant enzymes were usually studied in the responses to abiotic and biotic stresses, but not often for graft stress; in this study, we suggest grafting as stress because of, cut or wound stress, complete dark, and high humidity stress, especially in the first three days after grafting (healing response) [12,20]. The most important critical

period in the grafting healing process was at 2 and 3 days after grafting [51]. The results in both seasons showed that the activities of SOD, POD, and CAT were significantly different at the grafting healing process between polyploid watermelons (Figure 6). It has been reported already by [42,75] that high activities of POD and CAT, during the healing process have a high ability to scavenge reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in plants. H_2O_2 product when the plant is wounded or stressed and caused cell death [76].

Higher enzymatic activities and accumulation of phenolic compounds are typically associated with plant stress resistance [77]. Phenols contribute to the elimination of reactive oxygen species (ROS) [78]. In our results, the content of phenols was higher in Tetra than Di and Tri in all stages, but because the accumulation of phenols leads to incompatibility [25,26], a higher decrease in phenol contents were observed at later stages of grafting (15 DAG) (Figure 7). The decrement rates between 3 and 15 DAG in Tetra were (38.24, and 30.77%) in the first and second season, respectively, while in Di, the content of phenols increase at 15 DAG in the first season and decrease (27%) in the second season. With Tri, the decrement rates were (15, and 18.7%) at 15 DAG in the first and second season, respectively. These results can explain the high compatibility of Tetra and low compatibility in a Di. Also, our results are similar to [77], who found that the phenolic content and lignin content were higher in the compatible combination.

On the other hand, generally, carbohydrates are the source of energy in the plant, and the cotyledons are the source of carbohydrates in the seedlings [46,79]. The survival rate was positively correlated with an increase in starch content [47]. Our experiment to increase the percentage of carbohydrates before grafting by adding a solution of sucrose with different concentrations (0, 1, 2, and 3%) to the rootstocks, led to an increase in the accumulation of starch in the plant before grafting and this led to an increase in the survival rate, these results were identical with [46]. We applied sucrose to the rootstocks seedlings, and a higher survival rate was observed in the case of 2% sucrose application followed by a 3% sucrose application, in polyploid watermelon. With 2% sucrose application, survival rates were 74.23, 83.13, and 92.57 % in the first season and 74.24, 83.13, and 94.57 % in the second season in Di, Tri, and Tetra watermelon, respectively. Whereas with 3% sucrose application, the survival rates were 62.33, 86.6, and 89.07 % in the first season and 62.33, 78.6, and 87.07 % in the second season in Di, Tri, and Tetra

watermelon, respectively, with significant differences between polyploids. The accumulation of starch in the grafting union was positively increased with increasing the concentration of sucrose in the solution, as the treatment with a concentration of 3% recorded the highest rate of accumulation of starch, followed by 2% compared to the control, this results was Identical with [46] who conducted a similar experiment to increase the survival rate in watermelon grafted by splice method. The accumulation of starch in the graft union at the stage 3 DAG (the critical period in which the highest rate of energy is required to callus initiation and differentiation) was significantly higher in Tetra than in Tri and Diploid and were 2.13 and 1.77 times higher than Di in the first and second seasons, respectively, and 1.58 and 1.51 times higher than Tri in the first and second seasons, respectively. These results could explain the increase in the survival rate by increasing the concentration of sucrose in the solution and also could explain the reason for the increase in survival rate in tetraploid more than triploid and diploid because of its ability to store the highest rate of starch over triploid and diploid. These results are consistent with [48], who suggested that increasing carbohydrate concentrations in grafted tissues may increase grafting success. These results indicate that sucrose solution applied as a soil drench was taken up by rootstocks seedlings led to an increase in the accumulation of starch in the grafted plants and thus led to an increase in the survival rates in polyploid watermelon. Just as hormones are important in the process of compatibility and callus formation, so are carbohydrates [46-50]. And it has been proven by the experiment that increasing the percentage of carbohydrates led to an increase in the rate of grafting survival [46].

Overall, compatibility is a phenomenon that occurs through a combination of factors including higher IAA, starch contents and POD, CAT, SOD activities. All these factors are more or less linked to each other thus, making it easier for the plant to survive.

4. MATERIALS AND METHODS

4. 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 [80], 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 [10,81]. The grafting process was performed after 15 and 20 days after rootstock seeds sowing. The splice grafting method was used [46] (Figure S1). Rootstock seedlings were subjected to adaptation before and after grafting to increase the survival rate, as mentioned by [82,83].
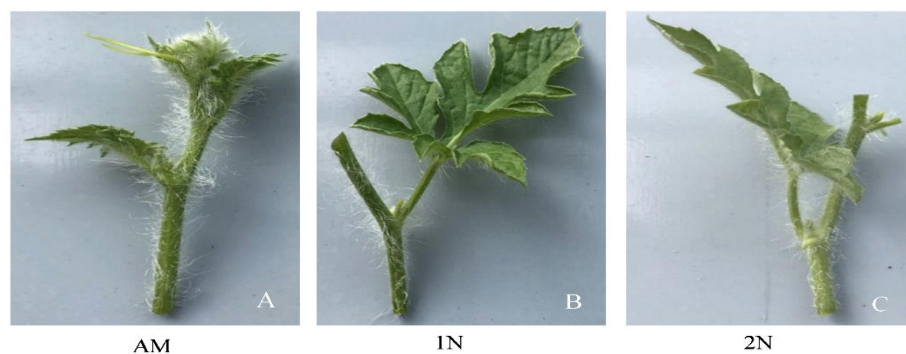

Figure 1. Three types of branches were taken from mother plants to use as scions in di, Tri, and tetraploid watermelon, **(A)** apical meristem (AM), **(B)** branch has one node, and one leave (1N), and **(C)** branch has two nodes (2N)

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

4.2 Sucrose application

Sucrose or water applications were applied as a soil drench to rootstocks seedlings according to the protocol established by [46]. Sucrose solution was applied in the morning at 2 and 4 days before grafting. Rootstocks seedlings were watered following common greenhouse practices on days when sucrose treatments were not applied. In this experiment, there were three replicates of 15 plants per treatment, in two consecutive seasons, the design was a randomized complete block, with three replications and three biological replications.

4.3. Data collection

4.3.1. Survival rates

Survive rates were investigated after 15 days using the formula [42].

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

4.3.2. Detection of Hormones Content by ELISA

Samples were collected from the graft union at 0, 3, and 15 days after grafting (DAG) with three biological replicates. The contents of endogenous Indole-3-acetic acid (IAA) and Zeatin Riboside (ZR) were measured with the Enzyme-Linked Immunosorbent Assay (ELISA) method (College of Agriculture and Biotechnology, China Agricultural University) with three biological replicates for each set of treatments. The determination of hormonal contents was performed as outlined by [30].

4.3.3. Assay for antioxidants enzymes activity and H_2O_2 contents

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

4.3.4. Quantification of biochemical at different stages

All experiments were carried out using starch assay kit/ YX-C-C400 for starch content estimation at 620 nm wavelength, lignin assay kit/ YX-C-B636 for lignin content using 280 nm wavelength, and phenol assay kit/ YX-C-A507 for total phenols content using 760 nm wavelength. (Sino best biological technology co, Ltd, Beijing, China) according to the manufacturer's instructions. Three biological replications from grafting union at 0, 3, and 15 DAG from three different plants for each replicate were collected for analysis.

4.3.5. Identification of genes involved in the compatibility mechanisms.

The protein sequences of genes involved in compatibility mechanisms were downloaded from the watermelon database http://cucurbitgenomics.org/organism/2. A total of 26 genes involved in compatibility mechanisms were identified based on the similarity index between protein sequences. The protein sequences for compatibility mechanisms in watermelon were downloaded from the 97,103 Watermelon Genome Database. MEGA 6 software was used to draw phylogenetic trees of selected genes. The clustalW tool was first used for the alignment. Afterward, the neighbor-joining method, with 1000 bootstrap replicates, was used [53,84]. The phylogenetic trees were constructed using protein sequences of the compatibility mechanisms.

4.3.6. Characteristics and structural analysis of genes responsible for compatibility mechanisms in watermelon

Gene names, accession number of genes, genomic lengths, coding sequences (CDS) lengths, protein sizes, and isoelectric points (pIs) and Mw (Da) were downloaded from http://cucurbitgenomics.org/organism/1 and ExPASy http://web.expasy.org/computepi/ databases. Gene Structure Display Server (GSDS), a web-based bioinformatics tool, was used to give a structural representation to selected watermelon genes responsible for sugars and organic acids metabolism and transporter genes, including upstream/downstream regions, coding sequences (CDS), and intron numbers were constructed [85].

4.3.7. Isolation of RNA and first-strand cDNA synthesis

Using a Total RNA Kit (Tiangene, China), we extracted the total RNA from the grafting union according to the manufacturer's instructions. Samples from grafting

unions were taken at three stages (0, 3, and 15 days after grafting). We detected RNA degradation and contamination on a 1% agarose gel. Using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), the quantity quality of RNA was checked according to the manufacturer's instructions. To carry out quantitative reverse-transcription PCR (RTq-PCR), cDNA was synthesized from RNA with M-MLV Reverse Transcriptase (Promega, USA) and diluted 20 ng/ul.

4.3.8. RTq-PCR expression analysis of genes involved in compatibility mechanisms

Genes linked to the compatibility mechanisms were selected from the watermelon genome database (http://cucurbitgenomics.org/organism/21). To check the expression patterns of selected genes in grafted watermelon at 0, 3, and 15 DAG, quantitative reverse transcription PCR (RTq-PCR) was performed. The entire data were analyzed using the 2-ΔΔCt method [86]. Three independent biological replicates were used for gene expression analysis [87]. Actin *"cla016178"* was used as a reference gene [53].

4.3.9. Experimental design and statistical analysis

Data were statistically analyzed using analyses of variance (ANOVA), with the Stat soft statistical package MSTATC software program (Michigan State University, East Lansing, MI, USA). Mean values of all data for all parameters were subjected to analysis of variance (ANOVA) by using SAS (version 9) computer software programs [SAS inst., 2002]. A least significant difference (LSD) was used to compare significant means at 5% probability level. Principal component analysis (PCA) was performed by using SIMCA software (version 14; Umetrics, USA).

5. Conclusion

Seedless watermelons seeds production faces many problems. The current study concludes that vegetative propagation by branches grafting could be used in Tri and Tetra watermelon vegetative propagation with high survival rates and thus making seed production easier and cheaper. An enigma of grafting union compatibility was addressed here. The high contents of hormones, starch and high activities of antioxidants in Tetra more than Di because of the genome duplication, which gave high compatibility and high survival rates of grafting. Grafting using branches can be a good alternative to promote vegetative propagation in Tetra watermelon. These findings add significant information to our existing knowledge of compatibility in watermelon by

ultimately helping in vegetative propagation, breeding watermelon varieties of desirable values.

Supplementary Materials:Figure S1: Splice grafting method steps in polyploid watermelon plants, **Figure S2:** Effect of Scion/rootstocks combinations on the survival of polyploid watermelon grafting using (AM) as a scion. (A): at 0 DAG, (B) 15 DAG, and (C): 30 DAG, DAG: Days after Grafting. **Figure S3**: Effect of Scion/rootstocks combinations on the survival of polyploid watermelon grafting using (2N) as a scion. (A): at 15 DAG, and (B) 30 DAG. **Figure S4:** Structure of genes involved in graft compatibility during the watermelon grafting process. Yellow boxes and black lines indicating exons and introns respectively, while blue boxes at both ends of each gene indicate untranslated regions (UTRs).**Table S1:** Genes linked to graft compatibility having high homology with other genes selected from different plant species. **Table S2:** Primers used for qRT-PCR in this study.

Author Contributions

KMO and WL conceived and designed the experiments. KMO performed the experiments. KMO and MJU analyzed the data and wrote the manuscript. HG, EM, WD, PY, HZ, SZ, XL, NH, CG, MA, and EL participated in the experimentations and analysis. MJU, MA, HZ, and WL edited the manuscript. All authors read and approved the last version.

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