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Response of Ancient and Modern Wheat Varieties to Biochar Application: Effect on Hormone and Gene Expression Involved in Germination and Growth

Marco Racioppi [1], Maria Tartaglia [1], José María De la Rosa [2], Mauro Marra [3], Elisa Lopez-Capel [4] and Mariapina Rocco [1,*]

[1] Department of Science and Technology, University of Sannio, 82100 Benevento, Italy; marco.racioppi91@gmail.com (M.R.); marytrt@live.it (M.T.); rocco@unisannio.it (M.P.R)
[2] Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Reina Mercedes Av., 10, 41012 Seville, Spain; jmrosa@irnase.csic.es (J.M.R)
[3] Department of Biology, University of Tor Vergata, 00133 Rome, Italy; mauro.marra@uniroma2.it (M.M)
[4] School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne NE1 7RU, UK; elisa.lopez-capel@newcastle.ac.uk (E.L.-C.)
* Correspondence: rocco@unisannio.it; Tel.: +39-0824-305168

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Abstract: Agriculture has changed dramatically due to mechanization, new technologies, and the increased use of chemical fertilizers. These factors maximize production and reduce food prices, but may also enhance soil degradation. Sustainable agricultural practices include altering crop varieties and the use of soil amendments to increase production, improve irrigation, and more effectively use fertilizers. Ancient and modern durum wheat varieties have been shown to be tolerant to conditions caused by climate change and increase production. Biochar soil amendments have been reported to increase crop yields, soil fertility, and to promote plant growth. However, results are variable depending on biomass source, application conditions, and crop species. This study evaluates the crop response of two contrasting durum wheat varieties on an Eutric Cambisol amended with beech wood biochar. Wheat varieties used are Saragolla, an ancient variety traditionally used in Southern Italy, and Svevo, a widely used commercial variety. The effect of biochar soil amendment on the expression of genes involved in the germination of these two varieties of wheat was determined using RT-PCR. The content of hormones such as gibberellins (GAs), auxins (IAA), and abscisic acid (ABA) was determined. Results demonstrate that biochar had a stimulatory effect on the growth performances of Svevo and Saragolla cultivars at the molecular level. This correlated to the promoted transcription of genes involved in the control of plant development. Overall, the presence of biochar as soil amendment improved the germination rates of both varieties, but the ancient wheat cultivar was better suited to the Eutric Cambisol than the commercial variety. This trend was also observed in un-amended pots, which may indicate better adaptability of the ancient wheat cultivar to withstand environmental stress than the commercial variety.

Keywords: wheat; biochar; germination; hormone; gene expression

1. Introduction

Cereals are one of the most popular sources of food for humans and animals. According to FAOStat, the global wheat production reached 715.9 million tons in 2013 [1], and durum wheat (*Triticum durum*) is the most widespread crop in the Mediterranean area. It is the exclusive raw material used for pasta production, and a basic product of the Mediterranean diet. The area cultivated

with durum wheat in Italy remained on an annual average of 1.6–1.8 million hectares [1], but the increased risk of land degradation due to unsustainable land practices and climate change could affect productivity [2].

The high yields of "modern" wheat cultivars, such as Svevo, require the use of a large amount of mineral fertilizers, chemical herbicides, and fungicides, leading to a greater risk of environmental pollution [3,4]. Increased public interest in this problem and growing consumer demand for healthier products, have led to a greater emphasis on crops grown under an integrated farm management approach to sustainable agricultural systems. Although ancient wheat cultivars provide high quality semolina, they have been progressively abandoned in favour of genetically uniform high-yield commercial varieties, as they do not adapt to intensive cultivation parameters. This reduction in biodiversity of modern varieties may enhance susceptibility to pathogens and disease; and decrease grain quality and productivity under adverse environmental conditions [4]. Traditional wheat cultivars, such as Saragolla, are expected to demonstrate greater adaptability and resilience as a result of better tolerance to diseases and more efficient capacity for using soil resources [3]. Therefore, ancient varieties could be the best choice to low-input and organic growing systems.

There is a need to develop more sustainable agricultural practices to avoid a decrease in soil fertility and organic carbon contents, and an increase in soil erosion [4]. Soil degradation in Mediterranean regions is particularly critical due to the climatic peculiarities of these areas, which can lead to permanent and irreversible degradation of soil quality and productivity. In restoration processes, the application of organic amendments is used for the creation of soil substrates with incipient structure and stable aggregates and to improve the biological functionality of the soil [5]. The use of biochar as soil amendment has been proposed as a sustainable strategy to improve crop productivity [6,7], control soil salinity [8], and mitigate global warming of degraded soils. Biochar has been reported to enhance plant physiological response [9] and improve plant adaptation to dry periods in biochar amended soils [6].

Biochar is rich in stable forms of C that decompose in soil at slower rate than untreated biomass residues [10]. The composition and properties of biochars are largely dependent on pyrolysis conditions and on the nature of the feedstock [11]. In general, the addition of biochar to a degraded soil improves its structure, increases porosity, decreases bulk density and enhances aggregation and water retention, reducing irrigation demand [12]. The biochar effect on soil properties influences crop growth [13], being more effective in degraded soils than in healthy ones [14]. For this reason, there is an increased interest in biochar applications for ecological restoration and carbon sequestration in nutrient-poor soils. Land management adaptations to enhance crop production in response to land degradation and potential climate change include the use of varieties resistant to heat shock and drought [7]. Crop germination, growth and yield depend on both genetic and environmental factors [15]. The balance of plant hormones is of fundamental importance in germination. There is evidence that biochar application can stimulate gibberellin, auxin, and brassinosteroid regulation, promoting plant growth [16]. Gibberellins are growth hormones that stimulate plant elongation, germination and flowering in cereal grains. French and Yyer-Pascuzzi [16] observed that biochar promotes growth partially through stimulation of the Gibberellins (GA) pathway. Although these is some evidence of the impact of biochar on crop production, the impact of biochar on plant growth and on the activity of enzymes and hormones involved in plant growth has not been fully investigated [17,18]. The mechanism of whether and how biochar soil amendment influences hormones and gene expression involved in crop germination remains largely unknown. Therefore, further research is needed to understand the signaling pathways on different plant species and cultivars within species [19]. The aim of this study is to investigate the effect of beech-wood biochar on germination performances, hormone level variations, and transcription of growth-related genes of a commercial (Svevo) and an ancient (Saragolla) wheat cultivar from Southern Italy in an irrigated soil from the Campania region in Italy. We hypothesized that biochar may play a key role on the stimulation of gibberellins in wheat. This knowledge is crucial for optimizing the use of biochar in wheat production and for breeding biochar-responsive wheat varieties.

2. Materials and Methods

2.1. Description and Analysis of Samples

Surface samples (0–25 cm depth) were collected from a traditional agricultural soil in Avellino province, within the Campania region (Southern Italy) classified as Eutric Cambisol (FAO classification). After decades of intensive cultivation of cereal crops the land was fallowed for a ten-year period (with no cultivation or fertilizer treatment). Wheat cultivation was recently reintroduced at this area under organic management practices. Soil samples were dried at 40 °C for 24 h and sieved (<2 mm) to remove roots, small branches and mosses. The biochar used in this experiment was produced by Verforfood (GREEN BIOCHAR S.C.A.R.L., Turin, Italy). It is a fine grain char (<0.251 mm) produced from hard beech (*Fagus sylvatica*) wood at a pyrolysis temperature of 550 °C. Soil and biochar pH was measured in triplicate in H_2O (1/2.5 *v*/*v*) using a pH meter (MM40 CRISON S.A, Barcelona, Spain)) after shaking for 60 min and overnight sedimentation as reported by Obia et al. [19]. Total carbon (C) and nitrogen (N) concentrations of soil and biochar samples were determined in triplicate by dry combustion (1000 °C) using a Perkin-Elmer 2400 series 2 elemental analyzer. Water Holding Capacity (WHC) and ash content of soil, biochar and 5% biochar amended soils were measured following the procedure described by De la Rosa et al. [20]. Briefly, samples were dried at 105 °C and subsequently heated in a muffle furnace (750 °C for 5 h). The ash percentage is the proportional weight of the remaining ash from the oven-dried weight sample. Soil, biochar, and biochar amended soils properties are shown in Table 1.

Table 1. Properties of biochar, soil and 5% biochar amended soils.

Sample	pH	% C	% N	% WHC	% Ash
Soil	5.62	3.1	0.16	35.9	88.0
Biochar	8.21	81.1	0.91	364.4	7.7
Soil + Biochar (5%)	7.10	n.a.	n.a.	53.4	n.a.

C: Total Carbon content (%); N: Total Nitrogen content (%); WHC: Water holding capacity (%); Ash: Ash content (%); n.a. = not analysed.

2.2. Seed Germination

Seeds of the T. durum cultivars Svevo (Agrisemi Minicozzi, Benevento, Italy) and Saragolla (SYNGENTA, Milan, Italy) were sterilized in 1% *w*/*v* sodium hypochlorite for 30 min and rinsed in distilled water to remove the excess of chemicals.

Svevo Control (SVC), Saragolla Control (SRC), and biochar amended soil treatments (SVB and SRB) were prepared by placing 100 seeds of Svevo and Saragolla seeds in 1 kg soil (dry weight and 1 kg soil mixed with 50 g biochar in pots (23 cm diameter and 20 cm height), respectively. The dose of biochar applied was 5% *w*/*w*, equivalent to about 25 t ha^{-1}, and within the range used for pot biochar studies [21]. Three pots per treatment were prepared, randomly placed, wetted with deionized water to 50% water holding capacity (WHC) [22], and kept at 4 °C for 24 h. Germination was monitored and recorded every 24 h for 7 days (after 24, 48, 72, 96, 120, 144 and 168 h) at a controlled environment chamber at 30 °C [23]. Seeds were rewetted and water content maintained at 50% WHC during the 7-day germination experiment.

2.3. Hormone Extraction and Analysis

In order to correlate the hormone content of the Svevo and Saragolla varieties with the effect of biochar treatment on their germination performances, seedlings of the two wheat cultivars, grown in control soils and soils amended with 5% biochar were collected three days after sowing. Abscisic acid (ABA), indoleacetic acid (IAA) and gibberellin A3 and A4 (GA) content in wheat seedlings was measured by HPLC [24]. Five wheat seedlings were collected from each pot and frozen at –80 °C. Wheat samples (2 g) were grinded to a fine powder and diluted in methanol (2.5 mL g^{-1} of fresh tissue). Each extract was centrifuged (16,000 rpm for 10 min at 4 °C). The supernatant was

concentrated under vacuum, a volume of deionized water was added to each sample and extracted with an equal volume of ethyl acetate. Aqueous and organic phases were separated by centrifugation (16,000 rpm for 2 min). The lower aqueous phase was transferred to a new tube, and then the pH of the solution was adjusted below 3 to keep all the hormones in protonated form. The upper organic phase was recovered, dried under vacuum and dissolved in 30 μL of methanol before analysis by reversed phase-HPLC. HPLC analysis was performed on a LC-20 Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-20AT quaternary gradient pump, SPD-M20A photo diode array detector (PDAD) and SIL-20 AH autosampler (20 μL injection vol). Plant hormones were separated on a Gemini-NX C18 column (250 × 4.5 mm, 5 μm; Phenomenex, Torrance, CA, USA), which was assembled with a Security Guard® (Phenomenex) pre-column and eluted with a gradient of acetonitrile (ACN) containing 0.1% *v*/*v* trifluoroacetic acid (TFA) in aqueous 0.1% *v*/*v* TFA at 45 °C; ACN ramped from 15% to 30% in 5 min, from 30% to 50% in 5 min, from 50% to 80% in 2 min, with a flow rate of 1.5 mL min^{-1}. Separated compounds were identified through their retention times, UV spectra and literature data by comparison with IAA (12886, Sigma-Aldrich, St Louis, MO, USA), GA3 (G7645, Sigma-Aldrich) and GA4 (G7276, Sigma-Aldrich) standards. These standard compounds were also used to build up calibration curves (in the range 5–200 μg/mL) at specific wavelengths (λIAA = 254 nm; λGAs = 205 nm; λABA = 254 nm). Gibberellin concentrations are reported as the sum of GA3 and GA4 content and results are shown as μg of hormone per gram of fresh tissue.

2.4. Data Analysis

The results of germination assay and HPLC-based hormone analysis are expressed as means ±S.D. The identification of significant differences was performed by analysis of variance (ANOVA), followed by the Student-Newman-Keulus test with a minimum level of significance of $p < 0.05$.

2.5. RNA Extraction, cDNAs Synthesis and Reverse Transcription-Quantitative PCR (RT-qPCR)

The effect of biochar addition over especific key enzymes of the biosynthetic pathway of GAs was analyzed through their expression in three-days germinated wheat seedlings of the Svevo and Saragolla varieties, by RT-qPCR.

A Sigma mirPremier microRNA isolation Kit was used to extract RNA from wheat leaf samples. RNeasy/QIamp columns and RNase-Free DNase set (QUIAGEN) were used to degrade genomic DNA and obtain an eluate of pure RNA. The extracted RNA was retrotranscribed to cDNA and stored at −20 °C. "ImProm-II Reverse Transcription System Kit" (Promega) and the "Mj mini thermal cycler" (BioRad) were used for retrotranscription, and samples were stored at −20 °C. The expression of some genes involved in germination, gibberellins biosynthesis, cell expansion and growth were analyzed by RT-qPCR. Gene primers (as shown in Table 2) were designed using the NCBI Primer Blast tool. "EvaGreen 2X qPCR MasterMix-R" (Applied Biological Materials) kit was used for RT-qPCR. The thermal cycler used "7300 Real-Time PCR System" was set to perform an initial denaturation at 95 °C for 1 min, an annealing phase of 9 min at 95 °C and 40 successive cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). Experiments were carried out in triplicate and the relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [25].

Table 2. List of primers used for real time PCR.

Primers	Sequences
β-ACTIN	F: TGGACTCTGGTGATGGTGTC R: CCTCCAATCCAAACACTGTA
BIN2	F: GAGATCTAAAGCCTCAAAATCTT R: TGGCTTCACCTTTAACGAGCT
PIN1	F: ATCATCTGGTACACGCTCAT R: GGGAACTGCTCGGTTGAT
TIP2	F: GATGACTCCTTCAGCTTGG R: GGCGAAGACGAAGATGAG
XTH	F:GCCCTTCGTCGCCTCCTAC R: CGGCACAACAACAACTAGTGGTAG
EXP A2	F: CCACCATGATGTGTTGTTCC R: AGTAGGAGTGGCCGTTGATG
TaCPS	F: GTATGCAAGCTTACCGCGTG R: ACCCCCACAAGAATGTCCTC
TaKS	F: CAGGCCGGGGAGAAATCTT R: TGAGACAGCTCATCTGGGGA
TaKO	F: CCGGCACCGAGATAGTCATC R: GAGCAAATCCAGCACCTCAT
TaGA20ox1a	F: CCATCCTCCACCAGGACAAC R: GAGCTCCATCCTCTGTCTGG

3. Results and Discussion

3.1. Effect of Biochar on the Germination of Svevo and Saragolla Seeds

Figure 1 shows germination results for Svevo and Saragolla seeds measured every 24 h during a period of 7 days at 30 °C. The application of 5% biochar in soil improved and accelerated the germination rate of both Svevo and Saragolla wheat varieties. The ancient cultivar, Saragolla, exhibited better germination rates than Svevo, both in germination percentage and precocity. The addition of biochar influenced the soil properties of this Eutric Cambisol from the Campania region of Italy by increasing its pH from 5.6 to 7.1 and the water retention from 35.9% to 53.4% (Table 1). The latter was probably due to biochar high WHC, porosity and organic carbon content. The liming effect of biochar probably facilitated the germination and development of wheat seedlings. This is in agreement with reported literature on biochar promotion of plant growth [16]. Therefore, biochar amendment on this agricultural Campanian soil could be associated with an improvement in soil structure, water retention, nutrient availability [12], and facilitated root development of wheat varieties.

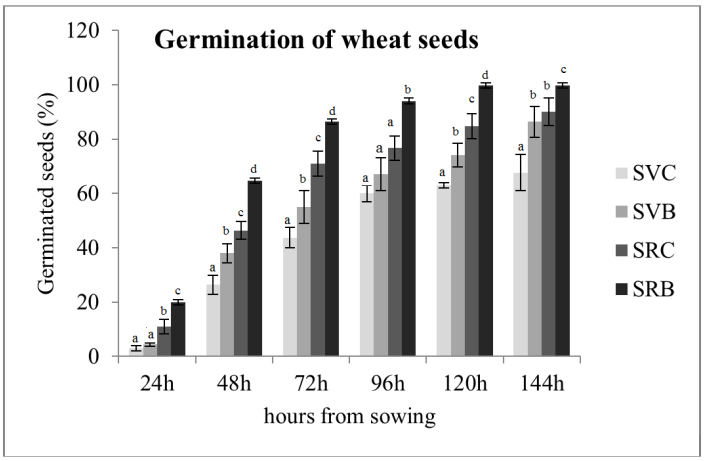

Figure 1. Effect of biochar on the germination of Svevo and Saragolla seeds. The histograms indicate the germination percentage for each sample; the vertical bars represent the standard deviation of mean of three replicates Svevo Control (SVC), Saragolla Control (SRC), Svevo Biochar (SVB) and Saragolla Biochar (SRB). Bars labeled with dissimilar letters are significantly different ($p < 0.05$).

3.2. Effect of Biochar on the Hormone Content of Svevo and Saragolla Seedlings

Figure 2 shows that the two varieties grown in soil without biochar (SVC and SRC) contained similar amounts of ABA while levels of 3-IAA increased significantly due to biochar addition. A significant increase of the GA content was also observed in biochar amended soils for both varieties. Overall, the total amount of GA was higher in Saragolla than Svevo cultivars, under both control and biochar amendment conditions. Germination and hormonal differences between the Saragolla and Svevo varieties support a model in which biochar application stimulates the GAs pathway in wheat, especially in Saragolla variety.

ABA and 3-IAA concentrations were significantly lower than those of GA. It is known that the stimulatory role of gibberellins (GA) in cereal grain have an interactive inhibitory effect on ABA transactivation activity, while the same does not affect GA activity [26,27]. These results demonstrate that, at a molecular level, the biochar soil amendment was able to markedly improve GA content of wheat seeds, thereby increasing their germination ability.

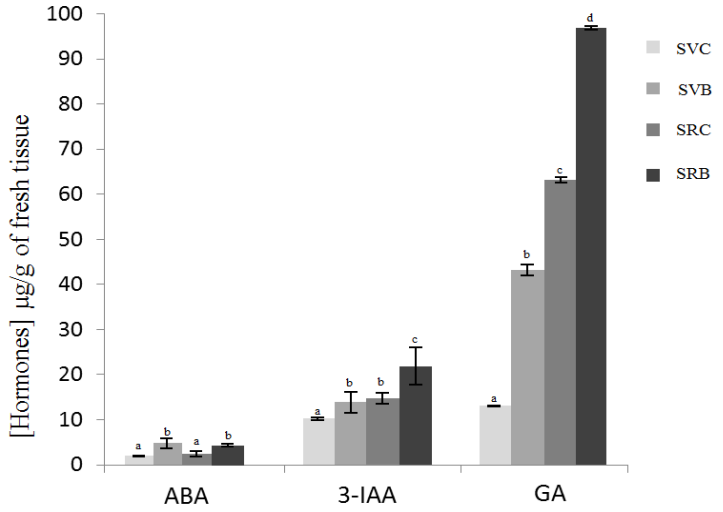

Figure 2. Hormone content of three-days germinated Svevo and Saragolla from sowing grown in soil amended with biochar. Histograms represent abscisic acid (ABA), auxins (3-IAA), and gibberellins (GA) contents (µg g^{-1} fresh weight) for each sample. Vertical bars represent the standard deviation of mean of three replicates. Svevo Control (SVC), Saragolla Control (SRC), Svevo Biochar (SVB) and Saragolla Biochar (SRB). Bars labeled with dissimilar letters are significantly different ($p < 0.05$).

3.3. Effect of Biochar on Gene Transcription

3.3.1. GA Biosynthesis Genes

Figure 3 shows a higher expression of ent-copalyl diphosphate synthase (TaCPS), ent-kaurene synthase (TaKS), ent-kaurene oxidase (TaKO) and GA 20-oxidase (TaGA20ox1a) in Saragolla than Svevo varieties. Moreover, the application of 5% wood biochar significantly increased the transcription of these genes in both varieties. In general, results confirm that the positive effect of biochar on the germination performance of wheat varieties is due to an increase in GA synthesis, which in turn depends on the stimulation of transcription of GA biosynthetic enzymes. Furthermore, the higher germination ability of the "ancient" Saragolla breed than the "modern" Svevo can be attributed to constitutively higher expression levels of GA biosynthesis enzymes. These results are in

agreement with those from French and Iyer-Pascuzzi [16]. Using the ga3ox1-3 Arabidopsis mutant for GA biosynthesis, they demonstrated that the GA pathway is involved in biochar-mediated plant growth promotion and hypothesized that the occurrence of hormone-like substances, such as karrikins, stimulate the GA pathway and stressed the importance of measuring GA levels in biochar-treated plants [28]. Our investigation demonstrates that biochar treatment stimulates wheat growth by increasing endogenous GAs concentration rather than by the release of hormone-like compounds. Figure 3 shows that biochar stimulates GA pathway by increasing endogenous gibberellins concentration, The increase of GA concentration (Figure 2), and GA biosynthetic enzymes (Figure 3); and accelerated germination (Figure 1) observed in biochar treated crops, provides conclusive evidence of the functional role of the GA pathway in biochar-induced plant growth promotion.

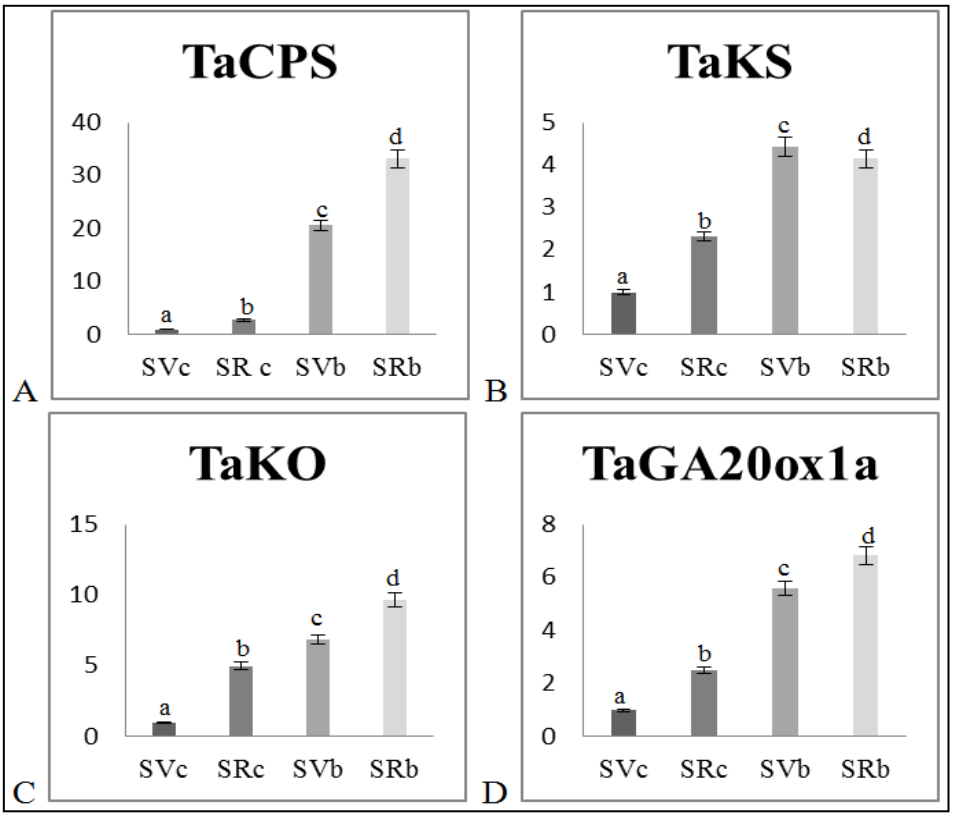

Figure 3. RT-qPCR analysis of the expression of the GA biosynthesis genes in Svevo and Saragolla cultivars upon biochar administration. (**A**) ent-copalyl diphosphate synthase (TaCPS); (**B**) ent-kaurene synthase (TaKS); (**C**) ent-kaurene oxidase (TaKO) and (**D**) GA 20-oxidase (TaGA20ox1a) for each treatment: Svevo Control (SVC), Saragolla Control (SRC), Svevo Biochar (SVB) and Saragolla Biochar (SRB). Bars labeled with dissimilar letters are significantly different ($p < 0.05$).3.3.2. Growth-Related Genes.

Viger et al. (2015) [29] reported the stimulation of the growth-promoting hormones auxin and brassinosteroid in Arabidopsis in plants treated with biochar. In order to determine whether the growth-promoting effect of biochar on Saragolla and Svevo cultivars could trigger auxin and/or brassinosteroid regulation, the expression of some key growth-related genes were analysed. Expansine A2 (EXP A2), xyloglucan endotransglicosylase (XTH), aquaporin 2 (TIP2), auxin efflux carrier (PIN 1) and brassinosteroid-insensitive 2 (BIN 2) gene expressions proteins were analysed by RT-qPCR on three-day old germinated samples. EXP A2, TIP2 and PIN 1 and XTH proteins participate at different levels of the growth-promoting pathway regulated by auxin; such as plant cell wall weakening (EXP A2), water transport (TIP2), auxin transport (PIN 1) and cell wall remodeling (XTH) [16,30]. Figure 4 shows that transcript levels of all of these proteins increased in both wheat cultivars in biochar amended soil treatments. This suggests that activation of the auxin responsive growth-promoting pathway can contribute to the stimulation of germination and growth observed

in the wheat cultivars with biochar treatment. Both in dicot and monocot XTHs are encoded by a multigene family and the expression of the individual XTH genes is differentially regulated by environmental stimuli [31] and growth-promoting hormones such as GAs and IAA [32]. XTHs are involved in the control of cell wall extensibility during growth stimulated by GAs and IAA [33] and the observed increase for XTH gene expression in the Saragolla cultivar upon biochar treatment is in accordance with the reported stimulation of growth and enhanced GAs biosynthesis of the cultivar after biochar treatment. Reasons for the absence of a similar increase in the Svevo cultivar are unclear. The BIN 2 gene encodes for a negative regulator of the brassinosteroid (BR) pathway which in plant regulates growth and development [34], interacting with other hormone pathways such as that activated by IAA [35]. Remarkably, levels of BIN 2 transcripts greatly decreased in both wheat cultivars after biochar soil amendment and particularly in the Saragolla cultivar, which showed the best germination and growth performance. This finding strongly suggests that the brassinosteroid-responsive pathway becomes activated upon biochar treatment and cooperates with GA and possibly IAA pathways to stimulate growth and development. Viger et al. [29] proposed that pH modification and increased K^+ supply and availability in biochar-amended soil could activate Ca^{2+} and ROS-mediated cell signaling, leading in turn to the stimulation of IAA and BR growth-promoting pathways. Our results show increased levels of endogenous GAs and suggest the activation of IAA and BR pathways, which are in line with the hypothesis of Viger et al. [29]. This provides mechanistic evidence of how biochar soil amendment influences gene expression involved in wheat germination.

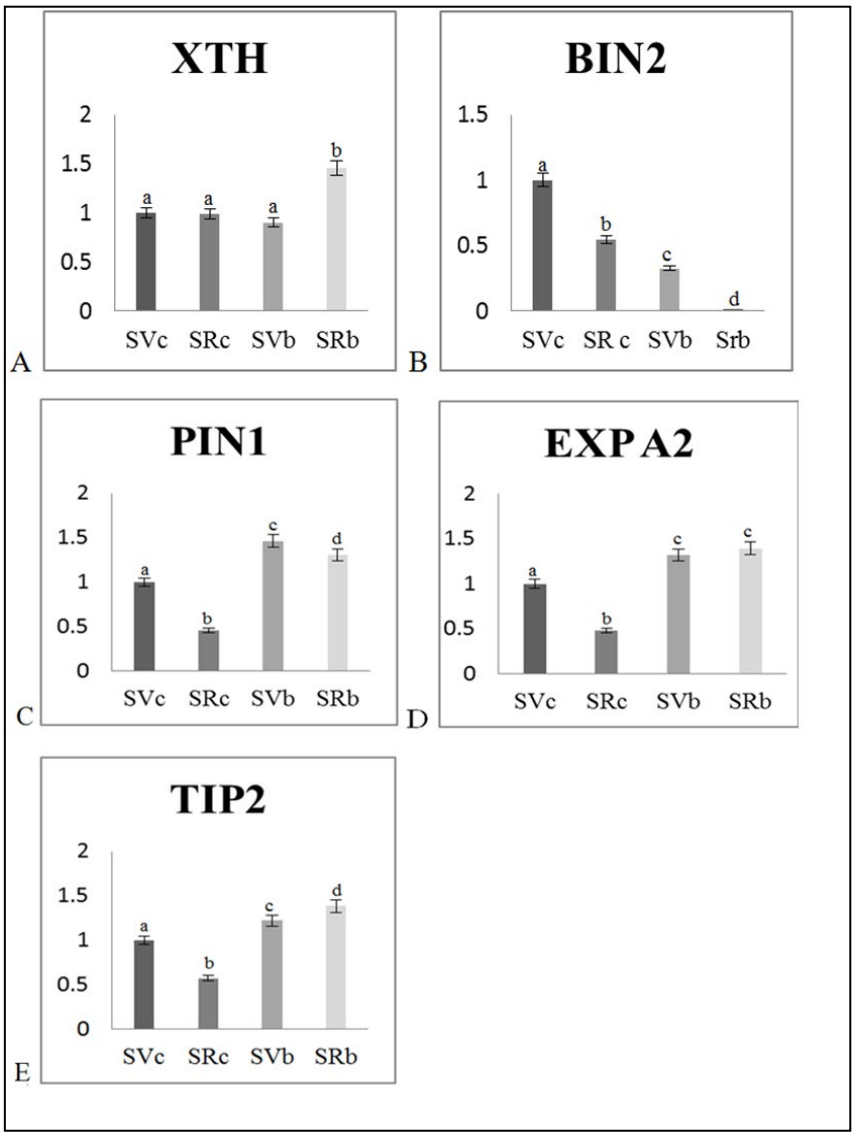

Figure 4. RT-qPCR analysis of the expression of growth-related genes in Svevo and Saragolla varieties upon biochar administration (**A**) xyloglucan endotransglicosylase (XTH), (**B**) brassinosteroid-insensitive 2 (BIN2), (**C**) auxin efflux carrier (PIN1); (**D**) expansine A2 (EXP A2), and (**E**) aquaporin 2 (TIP2) for the treatments Svevo Control (SVC), Saragolla Control (SRC), Svevo Biochar (SVB) and Saragolla Biochar (SRB). Bars labeled with dissimilar letters are significantly different ($p < 0.05$).4. Conclusions.

This study reports that the amendment of a typical agricultural Cambisol with 5% wood biochar enhanced seed germination and growth-hormone content of an "ancient" (Saragolla) and a commercial (Svevo) durum wheat variety. Biochar soil amendment had an effect on soil properties which could have facilitated wheat germination and root development. Agronomic data, hormone and gene expression analyses provided evidence of the growth performances and the stimulatory effect of biochar application on both varieties. At the molecular level, growth stimulation could be associated with an increase in GA levels and in transcripts of GAs biosynthesis genes. RT-qPCR results suggested that IAA and BRs pathways become activated upon biochar treatment, interacting with the GA pathway in the stimulation of growth and development of wheat cultivars. Although, the application of a 5% biochar soil amendment improved the germination and selected hormone content of both varieties, the "ancient" wheat cultivar achieved better results than the commercial variety for both untreated and to biochar amended cambisol. This study suggests that the use of biochar as a soil amendment and the use of traditional wheat varieties could improve wheat productivity in a sustainable way.

Author Contributions: M.P.R. (Mariapina Rocco), M.R. (Marco Racioppi) and J.M.R. (José M. De la Rosa) conceived the work. Conceptualization: M.P.R. (Mariapina Rocco); formal analysis: M.R. (Marco Racioppi) and M.T. (Maria Tartaglia); investigation: M.P.R. (Mariapina Rocco), M.R. (Marco Racioppi), M.T.; resources: M.P.R. (Mariapina Rocco) and M.M.; data curation: M.P.R. (Mariapina Rocco), M.R. (Marco Racioppi), M.T. and M.M.; Writing—original draft preparation: M.R. (Mariapina Rocco), M.R. (Marco Racioppi), M.T., J.M.R., E.L.-C. (Elisa López-Capel) and M.M. (Mauro Marra); Edition of the manuscript: M.R., E.L.-C. and J.M.R.; Visualization: M.M. All authors have read and agreed to the published version of the manuscript.

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