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Biochar: A Valid Additive to Enhance Kiwifruit In Vitro Proliferation

Samreen Nazeer [1], Valentina Morresi [2,3], Francesca Balducci [2], Leandra Leto [1], Andrea Di Fazio [1], Martina Cirlini [1], Anna Agosti [1,*] and Benedetta Chiancone [1,4]

[1] Department of Food and Drug, University of Parma, Viale Parco Area delle Scienze 27/A, 43124 Parma, Italy; samreen.nazeer@unipr.it (S.N.); leandra.leto@unipr.it (L.L.); andrea.difazio@studenti.unipr.it (A.D.F.); martina.cirlini@unipr.it (M.C.); benedetta.chiancone@unipr.it (B.C.)
[2] PROLAB, Società Agricola SALVI VIVAI s.s., Via Bologna 714, 44124 Ferrara, Italy; valentina.morresi@salvi.it (V.M.); franceca.balducci@salvivivai.it (F.B.)
[3] Department of Agricultural, Food, and Environmental Sciences, Marche Polytechnic University, 60131 Ancona, Italy
[4] Institute of Biophysics, National Research Council (CNR), Via Ugo La Malfa 153, 90146 Palermo, Italy
* Correspondence: anna.agosti@unipr.it

Abstract: Biochar, a by-product of agri-food waste, has shown benefits in plant growth and soil health. However, its use in vitro remains underexplored. This study investigates the impact of biochar supplementation in the culture medium, alone or in combination, with 6-benzylaminopurine (BAP), on kiwifruit (*Actinidia chinensis* var. *deliciosa*), cv. Tomuri proliferation. Kiwifruit explants were cultured on media enriched with 0, 4, or 6 g/L biochar, without or with BAP (0.2 mg/L), over two subcultures (SUB1 and SUB2). Parameters such as shoot and root number and length, fresh and dry weight, as well as plantlets' total phenolic content and antioxidant activity, were measured and analyzed. Biochar enhanced plantlets proliferation, particularly with BAP. In SUB1, at 4 g/L, biochar promoted shoot production (2.00 vs. 1.63) and their length (1.50 cm vs. 0.98), independently of the presence of BAP. The presence of biochar in the BAP-free media, favored rhizogenesis; particularly in SUB2, where on average, 5.58 roots per plantlets were recorded. Biochar increased the plantlets' total phenolic content and antioxidant activity, especially in BAP-free media. The addition of biochar as an additive to the culture medium during the kiwifruit in vitro proliferation phase could be a breakthrough outcome for the nursery sector.

Keywords: *Actinidia chinensis* var. *deliciosa*; agricultural waste; circular economy; growth regulators; in vitro tissue culture

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1. Introduction

Kiwifruit (*Actinidia chinensis* var. *deliciosa*) is a delectable and nutrient-rich fruit that is native to China [1]. It has taken the global market with its unique taste profile and abundance of vitamins and antioxidants [1]. Nowadays, the market demand for kiwifruit is continuously growing as well as the plant request, thus, the nursery sector is strategic to guaranteeing a high number of quality plants [2]; therefore, it is mandatory to develop efficient and sustainable propagation techniques.

Kiwifruit propagation can be achieved through sexual (generative) or asexual (vegetative) methods. However, commercial production overwhelmingly favors vegetative techniques due to several limitations of sexual propagation [3]. Firstly, generative methods lead to highly variable offspring due to genetic recombination during meiosis. Secondly, kiwifruit exhibits a dioecious nature, meaning separate plants produce only male or female

flowers. Seed-based propagation results in roughly 80% male and 20% female plants, significantly decreasing the chances of obtaining fruiting vines [3,4]. Thus, kiwifruit is mainly propagated agamically, through softwood or hardwood cuttings. However, due to the limited number of branches, the traditional cutting and grafting methods make it difficult to obtain large numbers of plants in a short time [5]. This challenge has spurred research into alternative propagation methods, with tissue culture emerging as a promising avenue. Pioneered by Harada's work in 1975 [6], this technique has been extensively explored using various *Actinidia* species and plant tissues [7–11]. Micropropagation emerges as a frontrunner for large-scale production of kiwifruit plants with desirable characteristics [5]. Micropropagation offers numerous advantages over traditional propagation methods; it enables the rapid multiplication of disease-free plants with true-to-type characteristics, independently of seasonal variations [12]. Conventionally, micropropagation relies on aseptic techniques and a basal nutrient medium. The culture medium composition is a key factor for the success of plant micropropagation; in fact, all of its components play a role in determining plant responses, particularly, the choice of type and concentration of growth regulators can trigger in vitro morphogenesis, leading to organogenesis, embryogenesis, etc. [12]. Among these regulators, 6-Benzylaminopurine (BAP) stands out as a commonly used cytokinin that promotes bud differentiation and shoot proliferation [13]. However, the optimal concentration of BAP can vary depending on the plant species, genotype, and type of explant [14].

Other than growth regulators, several studies are reporting the use of low-cost organic materials to partially or completely replace growth regulators in order to reduce plant production costs and the incidence of somaclonal variation, while maintaining high plant quality [15]. To reap the benefits of micropropagation, optimizing the in vitro culture conditions and testing additives of different origins for the culture medium is crucial for achieving successful results in micropropagation [16]. Woodchip biochar, a charcoal-like material obtained through the pyrolysis of organic matter [17,18], has assembled significant interest as a potential additive in plant tissue culture, due to its multifaceted benefits. Wiszniewska et al. [19] reported that the application of biochar as a supplement in culture medium enhances the micropropagation efficiency, reducing the accumulation of proline, betaines, and other stress-related phytohormones, i.e., abscisic acid and jasmonates, and improves the vigor and propagation of shoots, enhancing the level of gibberellins, salicylic acid, and benzoic acid. Incorporating biochar into micropropagation protocols can potentially make the process more sustainable, both because it is a valorization of waste and because it can allow a reduction in the use of growth regulators [20].

This study aims to evaluate biochar as an additive to the culture medium in order to investigate its role in improving kiwifruit proliferation, in combination or in alternative to BAP application. The cultivar studied was 'Tomuri', the primary pollinator for cv. Hayward, the most widely cultivated green kiwifruit variety globally [21].

2. Materials and Methods

2.1. Plant Material and In Vitro Culture Establishment

Branches were collected from virus-free mother plants of kiwifruit, cv. Tomuri, grown in screen-house at Centro Attività Vivaistiche (CAV) (Tebano, Emilia Romagna, Italy); cultures were established in vitro at the PROLAB of Società Agricola SALVI VIVAI, a nursery specialized in fruit plant production and the site of the experiment. Specifically, microcuttings were used as starting explants for the establishment of in vitro cultures, following the protocol set up by Mezzetti et al. [22,23]. Explants were surface disinfected for 15 min in a water solution with 1% (v/v) active chloride, prepared with a sodium hypochlorite (NaOCl) commercial product, and successively washed three times with sterile

water. Disinfected microcuttings were placed in plastic tubes containing the proliferation medium (PM) with the following composition: MS salts and vitamins 1× [24], added with 20 g/L of sucrose and 0.2 mg/L of BAP (Figure 1). pH was adjusted to 5.9, with NaOH 1 M [25]. Culture media were solidified with 6 g/L of agar (B&V srl., Gattatico, Italy) and sterilized in an autoclave, at 121 °C for 20 min. All chemicals were supplied by Duchefa Biochemie (Haarlem, The Netherlands).



Figure 1. Plastic tubes containing proliferation medium (PM) with MS salts, sucrose, and BAP for microcutting cultivation under sterile conditions.

After the establishment phase, plantlets were cultured in plastic jars and subjected to a proliferation phase, consisting of two subcultures, lasting 30 days each, in a growth chamber, at a constant temperature (24 °C ± 1), with a light intensity of 20 µmol m^{-2} s^{-1} and a 16-h photoperiod.

2.2. Experimental Design and Data Collection

In order to test the effect of biochar on kiwifruit proliferation, microcuttings were cultured for thirty days (SUB1), afterwards, after removing the basal callus, explants were sub-cultured on fresh PM for the following thirty days (SUB2). For both subcultures, the following culture media were tested: (1) 0BC-0BAP: PM, without BAP; (2) 4BC-0BAP: PM, without BAP, added with 4 g/L of BC; (3) 6BC-0BAP: PM, without BAP, added with 6 g/L of BC; (4) 0BC-0.2BAP: PM with 0.2 mg/L of BAP; (5) 4BC-0.2BAP: PM with 0.2 mg/L of BAP, added with 4 g/L of BC; (6) 6BC-0.2BAP: PM with 0.2 mg/L of BAP, added with 6 g/L of BC. Ten explants were placed in each sterile jar, three replications were disposed of per culture media.

The biochar used in this experiment was obtained through the pyro-gasification of various orchard residues (pruning and uprooting) at a target temperature typically set between 600 and 700 °C. The biochar was produced by AFE–Associazione Frutticoltori Estensi (Ferrara, Emillia Romagna, Italy) with an updraft gasifier manufactured by BiokW (www.BiokW.it accessed on 21 March 2024) (Trento, Italy), in compliance with the Italian regulation for biochar as a soil amendment, and is registered by AFE with the commercial

name "BiocharT". The physicochemical properties of the biochar used in this experiment are reported in Table 1.

Table 1. Characteristics of the biochar used in this study.

Particle Diameter (µm)	<500
Nitrogen (%)	0.6
Potassium (%)	0.8
Phosphorous (%)	0.07
Calcium (%)	2.7
Magnesium (%)	0.4
Sodium (%)	0.1
Total carbon (%)	62,5
Water holding capacity (%)	74.2
pH	9.85
Ash content (%)	22.8
pH	7.7
Electrical conductivity (dS m^{-1})	0.4
H/C	0.3

Other than continuous monitoring of the cultures to evaluate plant visual characteristics, the following parameters were measured at the end of SUB1 and SUB2: the presence of callus, number and length of shoots, number and length of roots, fresh weight, and dry weight. The presence of callus was measured visually on a scale from 0 to 4, giving 0 to plantlets without callus at their base and 4 to those who have regenerated it in the highest amount. Roots longer than 0.5 cm were scanned and analyzed by resorting to WinRHIZO™ 32 software version 2022b (Regent Instruments Inc., Quebec, QC, Canada). For dry weight determination, six plantlets were collected and weighed on an electric balance to obtain W1. After that, the dry weight (W2) was determined after 48 h, in an oven, at 72 °C.

The proliferation rate was calculated only for SUB1 since the plantlets recovered after the SUB2 were used for destructive measures and biochemical analysis. Biochemical analysis consisted of the evaluation of plantlet total (poly)phenolic content (TPC) and antioxidant capacity (AO).

2.3. Evaluation of Total (Poly)phenolic Content and Antioxidant Capacity

To evaluate the content of (poly)phenolic and antioxidant capacity of kiwifruit plantlets, the protocol described by Chiancone et al. [26] was followed. Plantlets were firstly lyophilized by a freeze dryer Lio-5P (5Pascal, Milan, Italy), and, after that, 1 g of dried substance was extracted with 20 mL of an ethanol/water solution (80/20), by shaking samples at 200 strokes/minute for 2 h at room temperature on a shaker (HS 501 digital shaker, IKA-Werke GmbH & Co., Staufen, Germany). The extracts were then centrifuged at 5000 rpm for 10 min at room temperature (Centrifugette 4206 centrifuge, Alc International, Pévy, France), and the supernatants were submitted for analysis. TPC was determined according to the Folin–Ciocalteau test, using gallic acid as the reference and measuring the absorbance at 760 nm by a JASCO V-530 spectrophotometer (Easton, MD, USA). For AO evaluation 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was applied, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard. In this case, the absorbance values at 517 nm were recorded.

All the analyses were repeated twice, and results were expressed on the dry matter content (DMC).

2.4. Statistical Analysis

Two-way analysis of variance (ANOVA) was used to statistically analyze data obtained at the end of both SUB1 and SUB2. Factors considered were "Biochar concentration" and "Presence of BAP"; Tukey's honest significance test (HSD) was used to separate the means at the 5% level of significance ($p < 0.05$) using the Systat Software (SYSTAT 13.1, Systat Software, Inc.; Pint Richmond, CA, USA).

3. Results

During SUB1, it was possible to carry out a first visual analysis of in vitro cultured explants (Figure 2). All the tested substrates guaranteed the survival of the plants, but differences in their development and proliferation were observed; moreover, the leaves of plantlets grown in biochar-enriched media seemed to be greener than those of control. In SUB2, plantlets grown on biochar-enriched medium showed leaf chlorosis; moreover, plantlets grown in culture media containing 6 g/L of biochar, showed signs of hyperhydricity. A better visual quality, including leaf color quality, habitus, and rooting, was detected in plantlets grown in culture media without BAP.

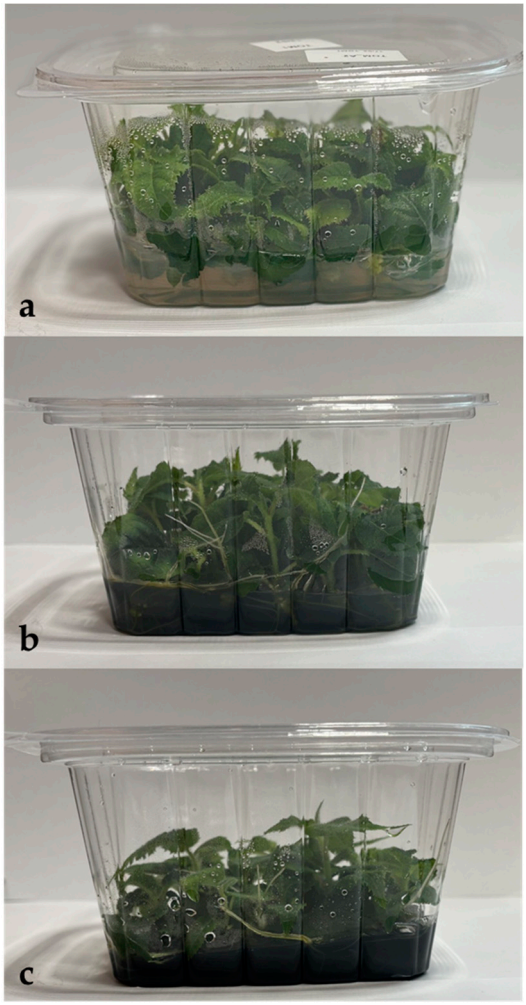


Figure 2. Kiwifruit, cv. Tomuri, shoots cultured in vitro on (**a**) the control medium and media enriched with biochar at (**b**) 4 g/L and (**c**) 6 g/L, at the end of the first subculture.

Specifically, in SUB1, the statistical analysis evidenced how the presence of biochar drastically reduced the presence of callus (Table 2); in fact, explants cultured on media without BC showed on average a callus presence higher than that of those grown on BC-

enriched medium (on the average 1.56 ± 0.27 vs. 0.2 ± 0.27). In SUB2, instead, callus was observed only at the base of explants grown on culture media containing BAP; for this reason, it was impossible to perform a statistical analysis of data.

Table 2. Influence of biochar and 6-benzylaminopurine on plantlets of kiwifruit, grown in vitro, at the end of first subculture.

[Biochar]	Presence of BAP	Callus	n° of Shoots	Shoots Length (cm)	n° of Roots	FW (g)	DW (g)
0BC	0BAP	1.45 ± 0.2744	1.40 ± 0.1604	0.98 ± 0.0959	1.13 ± 0.1250	0.51 ± 0.0449	0.22 ± 0.0559
	0.2BAP	1.68 ± 0.2875	1.85 ± 0.1581	1.00 ± 0.0480	1.00 ± 0.0000	0.98 ± 0.0868	0.24 ± 0.0491
4BC	0BAP	0.30 ± 0.0854	1.85 ± 0.1581	1.39 ± 0.0274	2.80 ± 0.7229	0.51 ± 0.0405	0.18 ± 0.0325
	0.2BAP	0.04 ± 0.0434	2.00 ± 0.2134	1.63 ± 0.0356	2.33 ± 1.3471	0.53 ± 0.0579	0.30 ± 0.0279
6BC	0BAP	0.27 ± 0.2920	1.60 ± 0.1777	0.79 ± 0.0176	3.33 ± 0.8606	0.50 ± 0.0372	0.22 ± 0.0111
	0.2BAP	0.19 ± 0.0779	2.10 ± 0.1857	1.20 ± 0.0201	2.64 ± 0.7063	0.45 ± 0.0295	0.26 ± 0.0303
Statistical analysis							
Factors		*p*	*p*	*p*	*p*	*p*	*p*
[Biochar] (BC)		<0.001	0.043	<0.001	0.092	<0.001	0.948
Presence of BAP (BAP)		0.804	<0.001	<0.001	0.525	<0.001	0.158
BC × BAP		0.412	0.985	<0.001	0.939	<0.001	0.249

Two-way ANOVA, Tukey's test ($p \leq 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. FW: Fresh weight, DW: Dry weight.

Kiwifruit plantlets responded differently in the two subcultures; specifically, in the SUB1, for the parameter "number of shoots", both factors individually exerted a significant influence (Table 2); in fact, plantlets grown on media enriched with 4 g/L of BC produced an n° of shoots statistically higher than those without BC (on the average 2.00 ± 0.12 vs. 1.63 ± 0.11), independently of the presence of BAP in the culture medium; moreover, a significantly higher number of shoots was obtained in plants grown in the presence of BAP rather than in those cultured in BAP-free medium (on the average 2.04 ± 0.10 vs. 1.62 ± 0.09) (Figure S1).

Conversely, at the end of SUB2, a significant interaction between the two factors was registered (Table 3); the presence of BAP was indeed irrelevant for the shoot production in plantlets grown on 0BC medium, while it induced the formation of a higher number of shoots if the culture medium was added with BC (4BC and 6BC) (Figure S2a). Finally, combining BC with BAP resulted in a significantly higher number of shoots per plant (Figure S2b).

Table 3. Influence of biochar and 6-benzylaminopurine on kiwifruit plantlets, grown in vitro, at the end of the second subculture.

[Biochar]	Presence of BAP	n° of Shoots	length of Shoots (cm)	n° of Roots	FW (g)	DW (g)
0BC	0BAP	2.30 ± 0.1527	2.15 ± 0.0108	3.60 ± 0.2477	0.47 ± 0.0073	0.09 ± 0.0011
	0.2BAP	2.10 ± 0.0999	3.35 ± 0.0118	0.00 ± 0.0000	1.83 ± 0.0012	0.14 ± 0.0012
4BC	0BAP	3.80 ± 0.1333	4.65 ± 0.0044	6.25 ± 1.2628	1.54 ± 0.0120	0.12 ± 0.0012
	0.2BAP	4.60 ± 0.1632	3.85 ± 0.0049	4.60 ± 0.2470	0.62 ± 0.0023	0.04 ± 0.0011
6BC	0BAP	3.40 ± 0.1000	4.84 ± 0.0046	6.75 ± 0.5803	1.39 ± 0.0425	0.10 ± 0.0007
	0.2BAP	4.10 ± 0.1632	5.16 ± 0.0066	4.75 ± 0.3170	1.86 ± 0.0118	0.03 ± 0.0012
Statistical analysis of the factors						
Factors						
[Biochar] (BC)		<0.001	<0.001	<0.001	<0.001	<0.001
Presence of BAP (BAP)		<0.001	<0.001	<0.001	<0.001	<0.001
BC × BAP		<0.001	<0.001	<0.001	<0.001	<0.001

Two-way ANOVA, Tukey's test ($p \leq 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. FW: Fresh weight, DW: Dry weight.

Regarding the length of shoots for SUB1 and SUB2, a significant interaction between the two factors was registered (Tables 2 and 3); in SUB1, the statistically longest shoots were obtained in presence of 4 g/L of BC, independently of the presence of BAP (Figure S3); while in SUB2, the longest shoots were obtained from plantlets cultured on medium enriched with both 4 g/L and 6 g/L of BC (Figure S4a). Particularly interesting is the result obtained for SUB2, within the 4BC treatment, where explants cultured on a medium BAP-free produced shoots statistically longer than those grown on BAP-enriched culture medium (Figure S4b).

For SUB1 and SUB2, kiwifruit plantlets started to produce roots after 20 days of culture. Regarding the number of roots, no significant differences were observed for any of the factors tested in SUB1 (Table 2), but a positive trend was evident; in fact, independently of the presence of BAP, as the concentration of biochar increased, an increasing n° of roots was registered (Figure S5). At the end of this subculture, none of the plantlets produced roots longer than 0.5 cm, thus this parameter was not included in the statistical analysis.

In SUB2, a significant interaction between factors was detected (Table 3), with plantlets grown in biochar-enriched culture media showing values higher than those grown on 0BC culture medium, independently of the BAP presence (Figure S6).

Since plants grown in the 0BC-0BAP medium did not produce any roots, it was not possible to include these data in the statistical analysis of root length; consequently, only roots of BC-treated plantlets were considered. When the combination of BC and BAP was tested, plantlets produced statistically longer roots in BAP-free media, independently of the biochar concentration (Figure S7).

The fresh weight was influenced by an interaction between the factors tested, in SUB1 and SUB2 (Tables 1 and 2). In the SUB1, in the absence of BAP, the addition of biochar to the medium did not influence the FW of kiwifruit plantlets (Figure S8); while, when BAP was added to the culture medium, the presence of BC was detrimental, with plantlets showing a FW statistically lower than those grown on BC-free medium (Figure S8).

In SUB2, in BAP-free medium, the presence of BC determined a significant increase of plantlet FW (Figure S9); while, in the presence of BAP, plantlets grown on 4BC showed a significant decrease (Figure S9).

Consequently, the dry weight of plantlets from the different culture media was recorded, and interestingly, it was observed that in SUB1, this parameter was not influenced by any of the factors considered (Table 2). In SUB2, an interaction between the factors studied was recorded (Table 3), and it was noticeable the same trend observed for fresh weight, confirming that, when BAP was not added to the culture medium, the addition of BC determined an increase in dry weight (Figure S10).

At the end of SUB2, the (poly)phenol content and antioxidant capacity in the plantlets were influenced by an interaction between the two factors (Table 4). In particular, it was observed that, in the absence of BAP, plantlets grown on a medium enriched with 6 g/L of biochar showed an increase in (poly)phenol content and antioxidant capacity; while, in the presence of BAP, the addition of BC caused a decrease in both TPC and AO (Figure S11a,b). Finally, the addition of BC led to an increase in TPC and AO, only in plantlets grown on the BAP-free medium; an opposite result was recorded for 0BC plantlets, in which statistically higher TPC and AO levels were observed in plantlets grown in the presence of BAP (Figure S11c,d).

Table 4. Influence of biochar and 6-benzylaminopurine on total (poly)phenolic content; and antioxidant activity of vitro-derived kiwifruit, at the end of the second subculture.

[Biochar]	Presence of BAP	TPC mg GAE/g	AO mg TEAC/g
0BC	0BAP	29.26 ± 3.0437	26.05 ± 3.2523
	0.2BAP	31.19 ± 2.9529	30.21 ± 2.0734
4BC	0BAP	26.19 ± 3.1100	21.02 ± 1.3760
	0.2BAP	16.33 ± 1.8456	11.01 ± 1.9808
6BC	0BAP	44.38 ± 5.8206	39.11 ± 0.5235
	0.2BAP	18.38 ± 0.2125	13.76 ± 2.1966
Statistical analysis			
Factors			
[Biochar] (BC)		<0.001	<0.000
Presence of BAP (BAP)		<0.001	<0.001
BC × BAP		<0.001	<0.001

Two-way ANOVA, Tukey's test ($p \leq 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. TPC: total (poly)phenolic content; AO: antioxidant activity.

4. Discussion

The influence of biochar on soil structure and biota has been widely studied, revealing significant benefits for plant and soil health, and overall ecosystem functioning [27,28]. However, while most research has focused on its application in field and soilless conditions, there remains a notable gap in its use for in vitro studies. Biochar has gained attention as a cost-effective and valuable solution for enhancing plant growth, primarily due to its ability to inhibit ethylene production and promote growth hormones in plant tissues [29,30]. Due to the small number of studies on the use of biochar in plant micropropagation, the discussion in this research will not only focus on in vitro applications of biochar, but also it will integrate findings from studies in vivo applications.

Over the years, micropropagation methods for kiwifruit have been developed to meet the growing market demand for this important fruit crop, focusing on minimizing callus formation and promoting better root development, resulting in a more efficient acclimatization process [31]. In this study, the presence of biochar in the culture medium drastically reduced the production of callus; this result is encouraging to improve the proliferation process, reducing plant production time.

Consistent with previous studies [32], the addition of BAP to the culture medium was essential for increasing the proliferation rate in kiwifruit micropropagation. Similarly, in this study explants grown in BAP-enriched media exhibited a significantly higher number of shoots across both subcultures (SUB1 and SUB2). The novelty of this study is the increased shoot proliferation, particularly evident in SUB2, due to the combination of BAP and biochar in the culture medium, highlighting the potential synergistic effect of these two culture medium additives.

In addition to shoot number, shoot length was positively influenced by the combination of BAP and biochar, in accordance with the findings reported by Nartop et al. [33]. In fact, in *Lavandula officinalis* L. micropropagation, the biochar addition determined an increase in shoot length [33]. However, as observed in SUB1, a higher biochar concentration (6 g/L) resulted in a reduction in kiwifruit shoot length. A comparable trend was reported in lavender, in which increasing biochar concentration from 0.5 to 2 g/L, a decrease in shoot length was observed [33]. In this study, this trend was not confirmed for SUB2, in which as the biochar concentration increased, the shoots obtained were longer.

The number and length of roots are key factors in determining plant survival during the acclimatization phase, therefore, in this study, these parameters were analyzed and compared with the data already available in the literature on other agronomically significant crops. As previously reported, BAP is generally detrimental to in vitro rhizogenesis; the same result was obtained in this study, particularly in SUB2, BAP addition determined a decrease in the root number. More importantly, biochar presence significantly promoted root emission, with its effect varying depending on its concentration. The positive role of biochar in rhizogenesis is supported by Di Lonardo et al. [29] and Santos et al. [34], who reported similar results in *Populus alba* L. and *Fragaria* × *ananassa* micropropagation.

Other than the number of roots produced, biochar also positively influenced root length. This effect has been observed across various plant species, from the model plant *Arabidopsis* in which the presence of biochar determined a 100% root length increase [35], to strawberry plants in which the biochar, added at the same concentration used in this study, contributed to enhance root length [34]. According to Xiang et al. [36], roots serve as interfaces between biochar particles and growing plants with 52% of studies reporting an increase in root length following biochar application.

A particularly noteworthy finding of this study is that plantlets grown in biochar-enriched BAP-free media developed a well-formed root apparatus. This result suggests the possibility of bypassing the in vitro rooting induction phase commonly employed in kiwifruit micropropagation [5,37,38], a step typically required by commercial propagation laboratories as that where the experiment was carried out.

Fresh and dry weight are key indicators of plantlet quality. In contrast with Nartop et al. [33], biochar positively influenced these parameters, particularly in the absence of BAP. This result is particularly relevant since it suggests that in the presence of biochar, plants can grow and proliferate, also in the absence of BAP; this outcome can be considered the first step of a potential strategy for reducing production costs while minimizing the risks of somaclonal variation, due to the use of this growth regulator.

Vitro-derived plantlets can be a wealthy source of bioactive compounds [39], thus, biochemical analysis of vitro-derived kiwifruit plantlets is essential. In this study, the presence of biochar stimulated the plantlets' secondary metabolism leading to an increase in both total (poly)phenolic content and antioxidant capacity. In literature, there is a lack of information about the influence of biochar on vitro-derived plant secondary metabolism; instead, it is demonstrated by Rosli et al. [40] and Zulfiqar et al. [41], respectively, in red lettuce and *Alpinia zerumbet* grown in vivo, that biochar enhances plant antioxidant defenses and mitigates oxidative stress by increasing phenolic acids concentration.

5. Conclusions

In order to improve the kiwifruit in vitro proliferation when it is carried out in a commercial laboratory, this research explores the impact of biochar supplementation in the culture medium, in combination, and as a replacement for BAP. Results evidence that biochar addition to the culture medium enhances kiwifruit plantlet proliferation, particularly, in the presence of 6-benzylaminipurine. Additionally, biochar supplementation positively influenced other key parameters, including the number and length of shoots and roots. The optimal biochar concentration for improving kiwifruit plantlet proliferation was identified to be 4 g/L, as it promoted plant proliferation while maintaining balanced root development. However, 6 g/L of biochar further improved specific parameters, such as shoot length in SUB2 and root length, suggesting that a higher concentration may be beneficial under certain conditions. From a sustainability perspective, integrating biochar, a by-product derived from agri-food waste, into the kiwifruit proliferation process, presents an opportunity to enhance efficiency while reducing costs. A particularly noteworthy

finding is the induction of rhizogenesis triggered by the biochar addition which could potentially eliminate the need for a rooting phase, thereby simplifying the micropropagation process. However, further studies are needed to confirm this possibility and refine biochar application strategies in the culture media. Considering the numerous existing applications of biochar, efforts should focus on expanding its use as an additive to the tissue culture media for the proliferation of other kiwifruit varieties and other agronomically important crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15040849/s1. Figure S1. Effect of biochar concentration and presence of BAP on the n° of shoots produced by kiwifruit microcuttings at the end of the first subculture. Within each factor, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S2. Effect of (a) biochar concentration, considering the presence/absence of BAP in the culture medium, and (b) the presence/absence of BAP per each biochar concentration tested, on the n° of shoots produced by kiwifruit microcuttings, at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S3. Effect of biochar concentration and presence of BAP on the length of shoots produced by kiwifruit microcuttings at the end of the first subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: A4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S4. Effect of (a) biochar concentration, considering the presence/absence of BAP in the culture medium, and (b) the presence/absence of BAP per each biochar concentration tested, on the length of shoots produced by kiwifruit microcuttings, at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S5. Effect of biochar concentration and presence of BAP on the number of roots in the first subculture. BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. Figure S6. Effect of biochar concentration and presence of BAP on the number of roots produced by kiwifruit microcuttings, at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S7. (a) Effect of biochar concentration and presence of BAP on the length of roots produced by kiwifruit microcuttings, at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). (b) Plantlet grown on a BAP-free medium, enriched with 4 g/L of biochar; (c) plantlet grown on a medium enriched with 0.2 mg/L of BAP and 4 g/L biochar and. Bars measure: 1cm. BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S8. Effect of biochar concentration and presence of BAP on the plantlets' fresh weight at the end of the first subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S9. Effect of biochar concentration and presence of BAP on the plantlets' fresh weight at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S10. Effect of biochar concentration and presence of BAP on the plantlets'

dry weight at the end of the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP. Figure S11. Effect of biochar concentration and presence of BAP on total (poly)phenol content (TPC) (a and c) and antioxidant capacity (AO) (b and d) of vitro-derived kiwifruit plantlets after the second subculture. Within each factor level, different letters indicate statistically significant differences. Two-way ANOVA, Tukey's test ($p < 0.05$). BC: biochar, BAP: 6-benzylaminipurine, 0BC: 0 g/L of biochar, 4BC: 4 g/L of biochar, 6BC: 6 g/L of biochar. 0BAP: 0 mg/L of BAP, 0.2BAP: 0.2 mg/L of BAP.

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