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Effect of Water Regime, Nitrogen Level, and Biostimulant Application on the Water and Nitrogen Use Efficiency of Wild Rocket [*Diplotaxis tenuifolia* (L.) DC]

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Abstract: The use of biostimulants in agriculture is an emerging technique that can contribute to improved production and resource use efficiency. This research was carried out in southern Italy to evaluate the biostimulating effects of seaweed extract (SW) and azoxystrobin (AZ) on wild rocket subjected to two water regimes (WRs) and three nitrogen levels (NLs), and grown in pots under unheated greenhouse conditions. The following treatments were compared: (i) two WRs: restoration of 100% (WR_{100}) and 50% (WR_{50}) of crop evapotranspiration; (ii) three NLs: 0 (N_0), 75 (N_{75}), or 150 (N_{150}) kg ha^{-1} of N; and (iii) three biostimulants (BSs): an untreated control (C), and the application of AZ or SW. This paper reports the effects on N uptake (N_{up}), N use efficiency (NUE), and water use efficiency (WUE). The following indicators of NUE were assessed: apparent recovery efficiency (RE), internal utilization efficiency (IE), partial productivity factor (PFPn) of N supplied, agronomic efficiency (AE), and physiological efficiency (PE). The following indicators of WUE were assessed: photosynthetic WUE (p_WUE), yield WUE (Y_WUE), biomass WUE (B_WUE), and irrigation yield WUE (IY_WUE). The indicators of NUE were affected differently by treatments. RE was 20% higher with SW. IE was higher with AZ. PFPn increased by 10.4 and 8.1% with AZ and SW, respectively. AE increased by 10.9 and 19.9% after applying AZ and SW, respectively. PE rose by 6.7 and 9.3% after applying AZ and SW. AZ and SW improved p_WUE, mainly under water deficit (interaction of WR × BS). With AZ application, Y_WUE, B_WUE, and IY_WUE were higher by 17.8, 13.8, and 19.3%, respectively, while the application of SW resulted in a smaller increase (9.5–7.7 and 9.9%). SW and AZ were shown to be effective through the moderate improvement of wild rocket's nitrogen and water use efficiency. The two biostimulants were more effective at improving p_WUE in water deficit conditions, proving to be particularly useful for farmers operating with water scarcity. Therefore, they can provide valuable support to farmers by improving the sustainability of resource use.

Keywords: seaweed; azoxystrobin; water deficit; WUE; NUE; N uptake

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1. Introduction

Different inputs required for the cultivation of any species represent a cost to farmers and contribute to the environmental impact of agricultural production. To maximize production, it is necessary to avoid both a deficit and surplus of resources. Usually, farmers tend to supply an excess of resources (i.e., water and nitrogen), with environmentally negative fallout. Therefore, the challenge is to optimize resource use efficiency, particularly water use efficiency (WUE) and nitrogen use efficiency (NUE), with the aim of reducing production costs and the environmental impact of agricultural production [1]. One of the possible solutions to this problem is the use of biostimulants, a new generation of products that are able to positively regulate the physiological processes of the plant and increase tolerance to abiotic stress [2–4]. Among the different classes of biostimulants, seaweed extracts are one of the most used in agriculture [5,6]. In addition, there are substances such as strobilurins that are normally used as fungicides, of which many authors have found complementary biostimulant effects [7–12].

Seaweed extracts (SW), mainly brown algae, represent a new promising tool for agriculture that can improve production, alleviate abiotic stresses (e.g., water deficit, salinity, high temperatures), and enhance resource use efficiency such as WUE and NUE, with positive effects on the environment [13–16].

The composition of commercial brown algae SW extracts can vary greatly depending on the raw material and the extraction method [13,17,18]. In fact, SW can contain, in different concentrations, a mixture of polysaccharides, phytohormones (auxins, abscisic acid, brassinosteroids, cytokinins, gibberellins), polyamines, phenolic compounds, vitamins, fatty acids, mineral nutrients, and a wide range of organic compounds [19–21]. In addition, several osmolytes (e.g., betaines) are present in SWs, which improve tolerance to heat and osmotic stress in plants [22,23].

Numerous studies are currently underway to understand the mode of action of the individual components present in SWs. For example, it was observed that SWs regulate the nitrate transporter gene 'NRT1.1′, which has a significant role in N uptake and N assimilation [24]. A more recent study has highlighted some possible mechanisms of SWs in the regulation of plant growth and development, such as the biosynthesis of new transporters for the absorption and assimilation of nutrients, hormonal homeostasis, the stimulation of photosynthetic activity, and stress tolerance [25]. In addition, SWs promote antioxidant stimulation by reducing lipid peroxidation occurring under abiotic stress and contribute to the scavenging of reactive oxygen species (ROS) [11,20,21,23].

Strobilurins are substances of natural origin that are produced by a group of fungi belonging to Basidiomycetes [e.g., *Oudemansiella mucida* (Schrad ex Fr) Hoehn and *Strobilurus tenacellus* (Pers ex Fr) Singer], which cause wood rot in numerous tree species [26]. Some structural variants have been produced from these substances (i.e., azoxystrobin, pyraclostrobin, kresoxim-methyl) used as broad-spectrum fungicides with very low toxicity toward humans and the environment. However, in addition to fungicidal activity, these compounds have also shown complementary biostimulant effects through an increase in the production and quality of various species [8,27].

Some studies have demonstrated the complementary properties of strobilurin-based crop protection products, which can improve yields and product quality while increasing resource use efficiency. In fact, these fungicides can influence some of the physiological processes of the plant by acting on the production of some phytohormones, such as abscisic acid (ABA), and some enzymes involved in oxidative stress, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) [8,11,28,29]. This gave rise to an improvement in gas exchange and WUE [7,11,30,31]. Furthermore, an increase in cell proliferation was observed [32], and an improvement in nitrogen metabolism with a reduction in the accumulation of nitrates was also observed, probably due to the stimulating action on nitrate reductase (NR) [27,33,34], as reported by several authors for wild rocket, spinach, and lettuce after the application of azoxystrobin [10,12,35,36].

Wild rocket [*Diplotaxis tenuifolia* (L.) DC] is a Brassicacea whose large-scale cultivation began about 25 years ago. Although there are still no official statistics relating to the surfaces and production of this crop, a recent estimate reported that, in Italy, the surface area cultivated by wild rocket is around 4800 ha [37]. The growing interest in this new vegetable derives both from its resilience to climatic adversity and from the qualitative characteristics of the product, which ensure high profitability [38,39].

The appreciation and consumption of wild rocket stems from its nutritional and organoleptic characteristics, which meet the growing demand for vegetables with high-value nutritional characteristics. This vegetable can be eaten raw as part of a salad or cooked as an ingredient in numerous recipes and has a unique aroma and a flavor with different intensity in terms of spiciness based on the cultivation conditions [40]. Wild rocket has high fiber, phenol, glucosinolate, ascorbic acid, carotenoid, sterol, fatty acid, and iron content, to which important bioactive properties are often attributed (e.g., anticancer and antioxidant properties) [41–44].

The characteristics of wild rocket have promoted its widespread cultivation even outside the area of origin in the Mediterranean, offering growers from all over the world the opportunity to have an interesting new species to be able to cultivate.

The growth of the market for ready-to-use salads, which in Italy are called 'IV gamma' salads, provides a further boost to the cultivation of wild rocket as it lends itself very well to this type of use. In fact, it is marketed as a 'baby leaf'.

Wild rocket can have several growth cycles thanks to the adventitious shoots that originate from the buds present on the roots. New shoots emerge at the base of the plant after the cutting of shoots 3–4 cm above the soil surface. Therefore, the same plants can provide two to five production cycles, even if the product with the best characteristics is obtained from the first harvest. To obtain good quality production, wild rocket requires adequate water availability throughout the growth cycle; water excesses, which can be very harmful, should be avoided. Furthermore, to obtain a high yield with tender leaves and good chlorophyll content, an adequate nitrogen supply must be ensured. Farmers tend to supply excessive quantities of nitrogen with negative repercussions on the environment and on some quality characteristics, such as leaf nitrate content.

The literature is lacking experimental evidence on the combined effect of water regime, nitrogen level, and the application of biostimulants, such as seaweed extracts and azoxystrobin, on wild rocket performance. On the basis of the scientific literature, it is hypothesized that wild rocket crops can also benefit from the application of the two biostimulants mentioned above through improved water and nitrogen use efficiency. Therefore, a study was conducted in which the effects of two water regimes, three nitrogen levels, and two biostimulants (azoxystrobin and seaweed extract) on the performance of wild rocket during two crop cycles were evaluated. This work reports the effects on water and nitrogen use efficiency strictly connected to the studied agronomic variables, the knowledge of which can be useful when trying to reach a compromise between obtaining excellent production performance and optimizing the efficient use of resources, which can translate into lower impact on the environment from wild rocket cultivation.

2. Materials and Methods

2.1. Characteristics of the Experimental Site

This trial was realized in the experimental greenhouse of the Mediterranean Agronomic Institute of Bari (CIHEAM-IAMB) (41°03′ N, 16°52′ E; 72 m a.s.l.) in the autumn–winter period of 2016–2017. The greenhouse had a 200 µm thickness EVA (ethylene vinyl acetate) film roof and was unheated. This site is characterized by a Mediterranean-type climate.

The wild rocket plants were grown in cylindrical pots (34 cm in diameter and 30 cm in height) and each of them was placed on a saucer. Each pot contained 20 L of soil (Lithic–Ruptic–Inceptic–Haploxeralfs type [45]) that had been taken from the fields near the greenhouse. The physical, chemical and hydrological characteristics of the soil are shown in Table 1.

Perlite at a 5:1 (v/v) ratio was added to the soil of the surface layer (0–5 cm depth) to avoid compaction resulting from watering.

Table 1. Main physical, chemical, and hydrological characteristics of the soil.

Parameters	Units	Values
Particle-size analysis		
Total sand (2 > ø > 0.02 mm)	(g 100 g^{-1})	20.6
Silt (0.02 > ø > 0.002 mm)	(g 100 g^{-1})	48.8
Clay (ø < 0.002 mm)	(g 100 g^{-1})	30.6
Chemical properties		
Total nitrogen (Kjeldahl method)	(g kg^{-1})	1.16
Available phosphorus (Olsen method)	(mg kg^{-1})	37.2
Exchangeable potassium (ammonium acetate method)	(mg kg^{-1})	345.4
Organic matter (Walkley Black method)	(g kg^{-1})	19.2
Total limestone (Dietrich-Fruhling method)	(g kg^{-1})	22.4
Active limestone	(g kg^{-1})	10.3
ECe (2:1)	(dS m^{-1})	0.35
ESP	(%)	1.3
pH (pH in H_2O)	-	7.5
Hydrological properties		
Field capacity	(cm^3 cm^{-3})	37.1
Wilting point (−1.5 MPa)	(cm^3 cm^{-3})	23.2
Bulk density	(kg dm^{-3})	1.23

ECe, saturation extract electrical conductivity ESP, exchangeable sodium percentage.

2.2. Climatic Trend

Inside the greenhouse, the average solar radiation during the cultivation period of wild rocket was about 9.5 MJ m^{-2} d^{-1}. This parameter had a quite stable trend around 7.5 MJ m^{-2} d^{-1} from transplantation until the end of January and then grew up to 14 MJ m^{-2} d^{-1} at the end of the second crop cycle.

The average daily minimum (T_{min}) and maximum (T_{max}) temperatures during the cultivation period were respectively equal to 7.3 and 15.4 °C, with a quite wide range of variations. In fact, T_{min} fluctuated between −2.1 and 16.9 °C, while T_{max} varied between 1.4 and 23.5 °C. During the first crop cycle, air temperatures were very low, especially in the first ten days of January. In fact, T_{min} was often below 0 °C and T_{max} did not exceed 5.5 °C. During the second crop cycle, T_{min} had a quite stable trend with values around 7 °C, while T_{max} increased until harvest with an average value of about 16 °C.

2.3. Treatments, Experimental Design, and Crop Management

The experiment involved the comparison of two water regimes (WRs), three nitrogen levels (NLs) and three biostimulants (BSs). WRs included the water restoration of 100% (WR_{100}) and 50% (WR_{50}) of the crop's evapotranspiration (ETc); NLs included 0 (N_0), 75 (N_{75}), and 150 (N_{150}) kg ha^{-1} of N supply; BSs included the untreated control (C), the application of a seaweed extract-based biostimulant (Bioproject SM23 Foliar-BioKimia® International S.r.l., Castel San Pietro Terme, BO, Italy) (SW), and the application of azoxystrobin (Ortiva®, Syngenta, Milano, Italy) (AZ). The treatments were arranged according to the split–split–plot experimental design with three replicates: WRs were the main plots, NLs the sub-plots, and BSs the sub-sub-plots which consisted of one pot.

P-K soil fertilization was performed in all treatments before transplantation as follows, and soil was then buried at a depth of 5–10 cm: 3.34 g pot^{-1} of P_2O_5 (as superphosphate) and 1.25 g pot^{-1} of K_2O (as potassium sulphate), corresponding to 70 kg ha^{-1} of both nutritional elements. Nitrogen fertilization in N_{75} and N_{150} treatments was split twice by adding 1.73 and 3.46 g pot^{-1} of 21% ammonium sulphate (40 and 80 kg ha^{-1} of N) to the soil before transplantation and 1.51 and 3.02 g pot^{-1} (35 and 70 kg ha^{-1} of N) at the very beginning of the second crop cycle.

The weeds were manually eliminated just after emergence to avoid competition with the wild rocket plants.

The seedlings of wild rocket were transplanted on 15 November 2016. Two crop cycles were carried out due to the regrowth capacity of this species after cutting. The first cycle ended on 17 January 2017, and the second one on 28 February 2017. For the transplant, seedlings at the 5-true leaf stage were used and raised in a nursery in polystyrene alveolate trays. A tuft of three seedlings had been grown in each alveolus. Five tufts per pot were transplanted after being suitably spaced. Thus, each pot contained a total of 15 plants.

BSs were sprayed (1.5 mL L^{-1} for SW and AZ, and deionized water for C) over the plants twice for each crop cycle 20, 50, 84, and 94 days after transplanting (DAT).

The two crop cycles took place without particular phytosanitary problems. Indeed, only one insecticide treatment (Confidor® 200 SL, Bayer CropScience S.r.l., Milano, Italy) was needed during the first crop cycle to control aphids (*Myzus persicae*).

The water balance method was used to measure the evapotranspiration (ET) of wild rocket. To achieve this, each pot was considered as a weighing lysimeter. The pots were weighed every 4–5 days at the same time (08:00–09:00 am). Average daily ET of the period between two successive weight measurements was obtained through the following equation [38]:

$$\mathrm{ET} = \frac{(\mathrm{W_n} - \mathrm{W_{n+m}}) + (\mathrm{W_I} - \mathrm{W_{Dp}})}{\rho_\mathrm{w}} \times \frac{1}{\mathrm{N_d}} \tag{1}$$

where:

ET is daily evapotranspiration (L);

W_n and W_{n+m} are two consecutive weights (kg) of the pot;

W_I is the supplied water (kg);

W_{Dp} is the drained water (kg);

ρ_w is the specific weight of the water (1 kg L^{-1});

N_d represents the number of days elapsed between two weight measurements.

The biomass increase between two weight measurements was considered negligible [46] and was not considered in the ET calculation procedure.

The waterings were planned on the basis of the threshold value of the readily available water reserve (p = 0.45) of the other *Brassicaceae* [47] subjected to the WR_{100} treatment. Fresh water with an electrical conductivity of 0.5 dS m^{-1} was distributed on the soil surface by hand to try and prevent leaf wetting.

Water balance calculations were carried out before the waterings to minimize water loss by percolation. In any case, when this occurred, the water collected in the saucers was used for the subsequent irrigation of the corresponding pot to recover any loss of nutrients due to leaching.

2.4. Yield, above Ground, and Root Biomass

The wild rocket harvest was carried out 63 and 105 DAT for the first and second crop cycle, respectively [12]. At the first collection, three tufts of plants were cut with a knife about 2 cm above the collar. The other two tufts were uprooted with the roots. The roots were cleaned of soil with running water and then dried with absorbent paper. Roots and above ground biomass (AGB) were separated by cutting them with a knife and then weighed. Of course, only three of the five initial plant tufts were present in the second growth cycle. At the second collection, all tufts were uprooted with roots and, as with the first collection, washed, dried, separated into roots and AGB, and weighed.

Total, marketable, and waste (leaves chlorotic, necrotic, and damaged by biotic and abiotic agents) yield at each harvest were assessed.

Dry above ground biomass (DAGB) and dry root biomass (DRB) were assessed by multiplying the total yield by the percentage of leaf dry matter, and root fresh weight by their percentage of dry matter [12].

For determination of the dry matter content of AGB (DM_{AGB}), a total product sample of approximately 150 g was used, while for the dry matter content of roots (DM_r) a smaller amount (20–40 g) was used. The plant material, after weighing, was placed in a thermoventilated oven at a temperature of 60 °C until it reached a steady weight (about 48 h). The DM_l and DM_r values obtained were expressed as a percentage of fresh weight.

2.5. Total Nitrogen of above Ground Biomass and Roots

The determination of total nitrogen in the AGB ($N_{\_AGB}$) and in the roots ($N_{\_r}$) was carried out by distillation according to the Kjeldahl method using a 1 g sample of dried and finely ground plant material to which 20 mL of sulfuric acid was added.

The methodology included hot (450 °C for two hours) oxidation of the organic nitrogen into ammonia nitrogen through reaction with sulfuric acid. The ammonium was then distilled in an alkaline environment. The total nitrogen content was expressed in g kg^{-1}.

2.6. Nitrogen Uptake and Nitrogen Use Efficiency

N uptake (N_{up}) was calculated at each harvest. This parameter was obtained from the product between dry biomass and the percentage of total N in the plant tissues.

At each harvest, the NUE was determined by taking into consideration the following five indicators [48]:

(1) The apparent recovery efficiency (RE);
(2) Internal utilization efficiency (IE);
(3) The partial productivity factor (PFPn) of the applied N;
(4) The agronomic efficiency (AE) of application;
(5) Physiological efficiency (PE).

The calculation of IE, PFPn, AE, and PE was carried out with respect to fresh (FW) biomass.

RE is most commonly defined as the difference in above ground biomass N uptake between fertilized and unfertilized crops relative to the amount of nutrient applied:

$$\mathrm{RE} = \frac{(\mathrm{U_x} - \mathrm{U_0})}{\mathrm{N_x}} \tag{2}$$

where:

U_x = N accumulated in the above ground biomass of the crop where a certain amount of nitrogen has been applied;

U_0 = N accumulated in the above ground biomass of the crop where no N was applied;

N_x = the amount of N added.

IE is defined as the yield in relation to the total uptake of the nutrient:

$$\mathrm{IE} = \frac{\mathrm{Y_x}}{\mathrm{U_x}} \tag{3}$$

where:

Y_x = yield of the crop with the application of a certain amount of nitrogen;

U_x = N accumulated in the above ground biomass of the crop where a certain amount of nitrogen has been applied.

PFPn represents the ratio between the weight of above ground biomass and the amount of N applied:

$$\mathrm{PFP_n} = \frac{\mathrm{Y_x}}{\mathrm{N_x}} \tag{4}$$

where:

Y_x = yield of the crop with the application of a certain amount of nitrogen (N_x);

N_x = the amount of nitrogen added.

AE represents the increase in yield with respect to N input. AE more closely reflects the direct impact on yield of the applied fertilizer and relates directly to the economic return. The calculation of AE requires knowledge of the yield obtained without nitrogen input:

$$\mathrm{AE} = \frac{(\mathrm{Y_x} - \mathrm{Y_0})}{\mathrm{N_x}} \tag{5}$$

where:

Y_x = yield of the crop with the application of a certain amount of nitrogen (N_x);

Y_0 = yield obtained without adding N;

x represents the amount of N added.

PE indicates the increase in yield in relation to the increase in N uptake by the above ground biomass:

$$\mathrm{PE} = \frac{(\mathrm{Y_x} - \mathrm{Y_0})}{(\mathrm{U_x} - \mathrm{U_0})} \tag{6}$$

where:

Y_x = yield of the crop with the application of a certain amount of nitrogen;

Y_0 = yield obtained without adding N;

U_x = N accumulated in the above ground biomass of the crop where a certain amount of nitrogen has been applied;

U_0 = N accumulated in the above ground biomass of the crop where no nitrogen was applied.

2.7. Water Use Efficiency

WUE was determined for each crop cycle, taking into consideration the four indicators described below [45].

When gas exchange was measured (61 and 104 DAT) (for the methodology of gas exchange measurements see Candido et al. [11]), photosynthetic water use efficiency (p_WUE) was calculated, which represents the ratio between net assimilation (A, μmol m^{-2} s^{-1}) and transpiration rate (T, mmol m^{-2} s^{-1}) at leaf scale [44]:

$$\mathrm{p\_WUE} = \frac{\mathrm{A}}{\mathrm{T}} \tag{7}$$

Yield WUE (Y_WUE), which represents the ratio between marketable yield (Y, kg ha^{-1}) and water loss by evapotranspiration (ET, m^3 ha^{-1}), was estimated as:

$$\mathrm{Y\_WUE} = \frac{\mathrm{Y}}{\mathrm{ET}} \tag{8}$$

Biomass WUE (B_WUE), which represents the ratio between DAGB (kg ha^{-1}) and ET (m^3 ha^{-1}), was calculated as:

$$\mathrm{B\_WUE} = \frac{\mathrm{DAGB}}{\mathrm{ET}} \tag{9}$$

Irrigation yield WUE (IY_WUE), which represents the ratio between marketable yield (Y, kg ha^{-1}) and seasonal irrigation volume (IV, m^3 ha^{-1}), was calculated as:

$$\mathrm{IY\_WUE} = \frac{\mathrm{Y}}{\mathrm{IV}} \tag{10}$$

2.8. Statistical Analysis

The datasets were tested according to the basic assumptions of analysis of variance (ANOVA). Normal distribution of the experimental error was verified by the Shapiro–Wilk test, while the common variance of the experimental error was examined by Bartlett's test.

The data collected in each crop cycle were subjected to ANOVA according to the split–split–plot experimental design with three replicates using SPSS 17. A three-way ANOVA procedure was performed considering all the factors as fixed and the replicates as random. Mean values were separated with the Student–Newman–Keuls (SNK) test ($p = 0.05$).

3. Results

3.1. Nitrogen Uptake and Nitrogen Use Efficiency

The total N content of the above ground biomass ($N_{\_AGB}$) and of the roots ($N_{\_r}$) of wild rocket was not affected by irrigation regimes or biostimulants, though it was influenced by the N level (Table 2). As the N supply increased, a progressive increase in the two parameters was observed which, as an average of the two collections, went from 3.7 and 3.0% of N_0 to 4.9 and 4.1% of N_{150}, respectively.

Table 2. Effect of water regime, nitrogen level, and the application of biostimulants on the total N content of above ground biomass ($N_{\_AGB}$) and roots ($N_{\_r}$) and on the N uptake of above ground biomass (N_{up_AGB}) and roots (N_{up_r}) of wild rocket for the two crop cycles. WR_{100} = restoration of 100% of ETc, WR_{50} = restoration of 50% of ETc; N_0 = 0 kg ha^{-1} of N, N_{75} = 75 kg ha^{-1} of N, N_{150} = 150 kg ha^{-1} of N; C = control; AZ = application of azoxystrobin; SW = application of a seaweed extract-based biostimulant.

Treatments	1st Crop Cycle				2nd Crop Cycle			
	$N_{\_AGB}$ (%)	$N_{\_r}$ (%)	N_{up_AGB} (g m^{-2})	N_{up_r} (g m^{-2})	$N_{\_AGB}$ (%)	$N_{\_r}$ (%)	N_{up_AGB} (g m^{-2})	N_{up_r} (g m^{-2})
Water regimes (WRs)	ns	ns	*	*	ns	ns	*	*
WR_{50}	4.3	3.3	3.8	1.8	4.0	3.3	2.6	1.5
WR_{100}	4.6	3.7	5.2	2.3	4.4	3.7	4.1	1.9
Nitrogen levels (NLs)	*	*	**	*	*	*	**	*
N_0	3.9 b	2.9 b	2.7 c	1.5 b	3.5 b	3.0 b	1.2 c	1.2 b
N_{75}	4.4 ab	3.5 ab	4.6 b	2.0 ab	4.3 ab	3.5 ab	3.5 b	1.9 a
N_{150}	5.0 a	4.1 a	6.2 a	2.8 a	4.8 a	4.0 a	5.4 a	2.0 a
Biostimulants (BSs)	ns	ns	ns	ns	ns	ns	ns	ns
C	4.4	3.4	4.3	1.9	4.0	3.4	3.1	1.6
AZ	4.4	3.5	4.8	2.2	4.3	3.6	3.6	1.7
SW	4.5	3.5	4.4	2.1	4.3	3.6	3.3	1.8
All interactions	ns	ns	ns	ns	ns	ns	ns	ns

ns, *, ** indicate non-significant or significant F-test at $p < 0.05$ and $p < 0.01$, respectively. Different letters indicate significantly different values according to the SNK test ($p = 0.05$).

On the other hand, N_{up} by AGB (N_{up_AGB}) and roots (N_{up_r}) was influenced not only by N level, but also by water regime. In particular, in WR_{50}, N_{up_AGB} and N_{up_r} were lower (as the average of the two collections) by 31.8 and 21.4%, respectively, compared to WR_{100} (Table 2).

With the increase in N level, N_{up} rose progressively, reaching an average increase of 240.0 (N_{up_AGB}) and 76.7% (N_{up_r}) in N_{150}.

The different indicators of NUE were influenced differently by the compared treatments (Table 3). RE was influenced by WRs and BSs. In particular, this parameter was, on average, 37.1% lower in WR_{50} compared to WR_{100}. Furthermore, in SW, it was on average 20% higher than in the control, while in AZ there were no relevant variations. IE was affected by NLs and BSs. The value of this parameter, in particular, decreased as the N level rose and, limited to the first harvest, was higher with AZ. The PFPn of the applied N decreased on average by 31.6% with the reduction in water availability and by 29.5% with the higher N level, while it increased by 10.4 and 8.1% with the application of AZ and SW, respectively. The AE of the N application dropped by 35.9% with reduced water availability, decreased by 11.7% with the highest N input, and rose by 10.9 and 19.9% by applying AZ and SW, respectively. PE decreased by 9.1% between N_{75} and N_{150}, though was instead increased by 6.7 and 9.3% after applying AZ and SW.

Table 3. Effect of water regimes, N levels, and biostimulants on some indicators of the N use efficiency (NUE) of wild rocket for the two crop cycles: apparent recovery efficiency (RE), internal utilization efficiency (IE), partial productivity factor (PFPn), agronomic application efficiency (AE), and physiological efficiency (PE). FW = fresh weight; WR_{100} = restoration of 100% of ETc; WR_{50} = restoration of 50% of ETc; N_0 = 0 kg ha^{-1} of N; N_{75} = 75 kg ha^{-1} of N; N_{150} = 150 kg ha^{-1} of N; C = control; AZ = application of azoxystrobin; SW = application of a seaweed extract-based biostimulant.

Treatments	RE (g N_{up} g^{-1} N)	IE (g FW g^{-1} N_{up})	PFP_n (g FW g^{-1} N)	AE (g FW g^{-1} N)	PE (g FW g^{-1} N_{up})
			1st Crop Cycle		
Water regimes (WRs)	*	ns	**	**	ns
WR_{50}	0.39	188.8	151.5	66.7	166.8
WR_{100}	0.55	184.8	205.2	94.4	172.6
Nitrogen levels (NLs)	ns	*	**	*	*
N_0	-	193.7 a	-	-	-
N_{75}	0.49	189.3 a	218.3	88.0	177.8
N_{150}	0.45	177.4 b	138.3	73.1	161.6
Biostimulants (BSs)	*	*	*	*	*
C	0.43 b	178.3 b	166.5 b	69.3 c	162.9 c
AZ	0.42 b	203.0 a	185.1 a	81.9 b	168.8 b
SW	0.55a	179.1 b	183.3 a	90.5 a	177.4 a
All interactions	ns	ns	ns	ns	ns
			2nd Crop Cycle		
Water regimes (WRs)	*	ns	**	**	ns
WR_{50}	0.45	187.3	126.3	81.9	181.6
WR_{100}	0.82	192.4	201.4	146.5	180.4
Nitrogen levels (NLs)	ns	*	**	*	ns
N_0	-	198.4 a	-	-	-
N_{75}	0.66	186.2 b	184.1	118.0	180.8
N_{150}	0.61	185.0 b	143.6	110.4	181.3
Biostimulants (BSs)	*	ns	*	*	*
C	0.59 b	188.7	155.7 b	109.5 b	170.0 b
AZ	0.64 ab	191.1	170.7 a	113.6 ab	186.6 a
SW	0.66 a	189.9	165.2 a	119.5 a	186.4 a
All interactions	ns	ns	ns	ns	ns

ns, *, ** indicate non-significant or significant F-test at $p < 0.05$ and $p < 0.01$, respectively. Different letters indicate significantly different values according to the SNK test ($p = 0.05$).

3.2. Water Use Efficiency

In general, the values of the WUE indicators varied significantly in relation to the compared treatments (Table 4, Figure 1).

As water deficit was more contained in WR_{50} (p = 50, 61 DAT), an increase in p_WUE of 19.6% was recorded compared to $WR_{100.}$ However, in conditions with more severe water deficits (p = 90, 104 DAT), p_WUE was not influenced by water regime (Figure 1).

In addition, p_WUE increased progressively with increasing N level, especially at 104 DAT. The application of AZ and SW resulted in an average increase in p_WUE of 25.2% and 13.3%, respectively. The increase of the latter parameter with BSs occurred mainly in conditions of water scarcity (interaction of WRs x BSs) (Figures 1 and 2).

Table 4. Effect of water regimes, nitrogen levels, and the application of biostimulants on (i) water use efficiency in relation to marketable yield (Y_WUE, kg m^{-3}) and above ground dry biomass (B_WUE, kg m^{-3}) and (ii) irrigation water use efficiency in relation to marketable yield (IY_WUE, kg m^{-3}) for wild rocket from two crop cycles. WR_{100} = restoration of 100% of ETc; WR_{50} = restoration of 50% of ETc; N_0 = 0 kg ha^{-1} of N; N_{75} = 75 kg ha^{-1} of N; N_{150} = 150 kg ha^{-1} of N; C = control, AZ = application of azoxystrobin, SW = application of a seaweed extract-based biostimulant.

Treatments	1st Crop Cycle			2nd Crop Cycle		
	Y_WUE	B_WUE	IY_WUE	Y_WUE	B_WUE	IY_WUE
Water regimes (WRs)	ns	ns	**	*	ns	*
WR_{50}	12.3	1.5	17.1	9.6	1.2	11.4
WR_{100}	11.4	1.3	11.6	8.3	0.9	8.9
Nitrogen levels (NLs)	**	*	**	**	*	**
N_0	7.4 c	1.0 c	9.0 c	3.4 c	0.5 c	3.9 c
N_{75}	12.4 b	1.5 b	15.1 b	9.2 b	1.2 b	10.4 b
N_{150}	15.7 a	1.8 a	18.9 a	14.3 a	1.6 a	16.1 a
Biostimulants (BSs)	*	*	*	*	*	*
C	10.8 b	1.3 b	13.0 b	8.3 b	1.0 b	9.3 b
AZ	12.7 a	1.4 ab	15.5 a	9.8 a	1.2 a	11.1 a
SW	12.1 a	1.5 a	14.6 a	8.9 b	1.1 ab	10.0 ab
All interactions	ns	ns	ns	ns	ns	ns

ns, *, ** indicate non-significant or significant F-test at $p < 0.05$ and $p < 0.01$, respectively. Different letters indicate significantly different values according to the SNK test ($p = 0.05$).

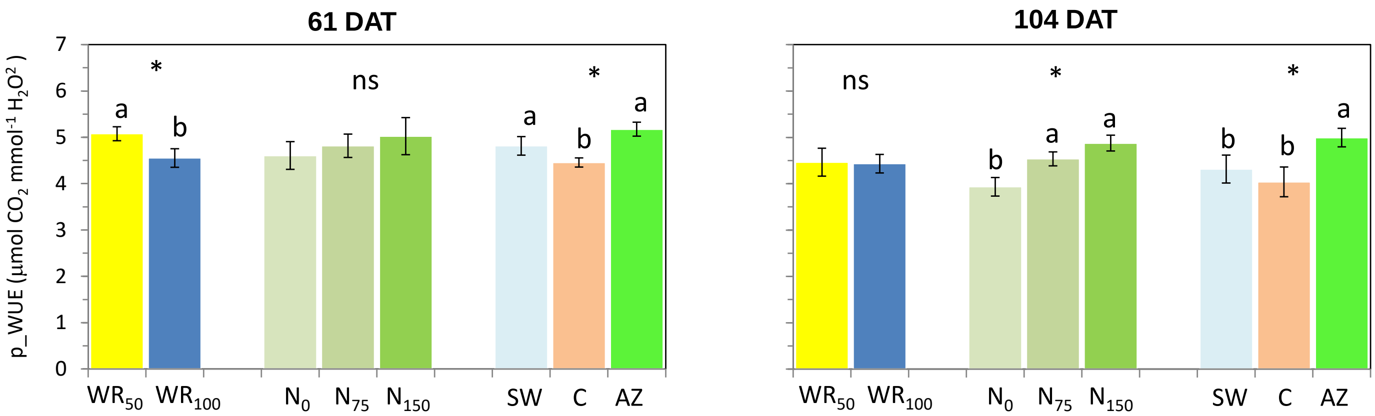

Figure 1. Effect of water regimes, nitrogen levels, and the application of biostimulants on the photosynthetic water use efficiency (p_WUE) of wild rocket 61 and 104 days after transplantation (DAT). WR_{100} = restoration of 100% of ETc; WR_{50} = restoration of 50% of ETc; N_0 = 0 kg ha^{-1} of N; N_{75} = 75 kg ha^{-1} of N; N_{150} = 150 kg ha^{-1} of N; C = control; AZ = application of azoxystrobin; SW = application of a seaweed extract-based biostimulant. The vertical bars indicate the standard deviation. ns, * indicate a non-significant or significant F-test at $p < 0.05$. Different letters indicate significantly different values according to the SNK test ($p = 0.05$).

Y_WUE, B_WUE, and IY_WUE, as the mean of two harvests and treatments compared, reached values of 10.4, 1.2, and 12.2 kg m^{-3}, respectively.

All the studied treatments influenced three WUE parameters. In particular, under water deficit, Y_WUE increased slightly, B_WUE did not change significantly, and IY_WUE increased by an average of 44.7%. The above parameters rose greatly with the increase in N level, especially in the second collection when, between N_0 and N_{150}, Y_WUE, B_WUE, and IY_WUE increased by more than 300, 200, and 300%, respectively. With AZ application, Y_WUE, B_WUE, and IY_WUE were higher by 17.8–13.8 and 19.3% (as an average of the collections), while the application of SW resulted in a smaller increase (9.5–7.7 and 9.9% respectively).

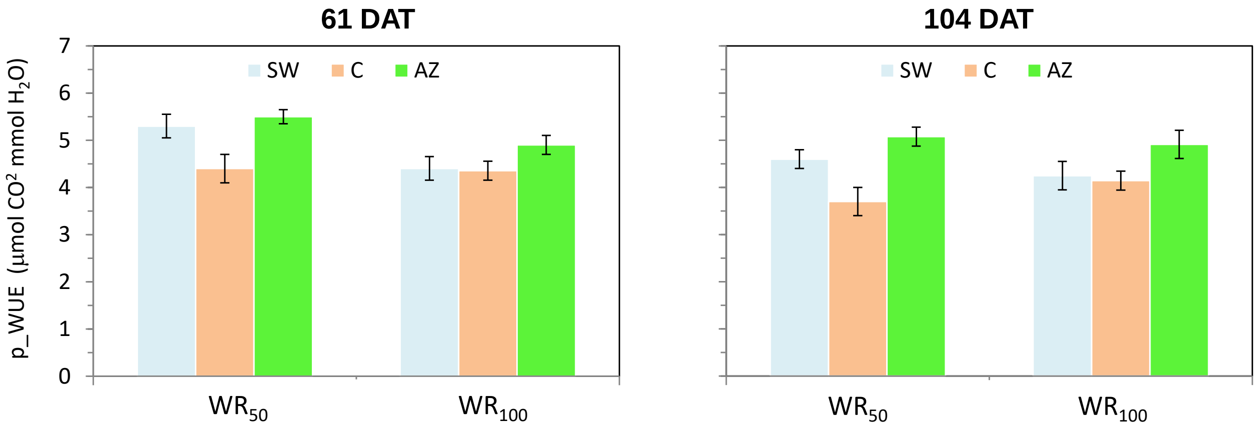

Figure 2. Interaction between water regime and treatments with biostimulants on the photosynthetic water use efficiency (p_WUE) of wild rocket 61 and 104 days after transplantation (DAT). WR_{100} = restoration of 100% of ETc; WR_{50} = restoration of 50% of ETc; C = control; AZ = application of azoxystrobin; SW = application of a seaweed extract-based biostimulant. The vertical bars indicate the standard deviation.

4. Discussion

4.1. Nitrogen Uptake and Nitrogen Use Efficiency

The percentage of N contained in the wild rocket above ground biomass was not influenced by irrigation regime, in contrast to the results reported by Candido et al. [9] in which a lack of water had led to a reduction in N percentage, as also observed by Santamaria et al. [49] for *D. tenuifolia* and *E. vesicaria* in hydroponics.

The water deficit reduced the amount of nitrogen removed from the crop. This is mainly attributable to the difference in total biomass produced in the two irrigation treatments. Furthermore, wild rocket NUE worsened when water supply was limited, as already observed in lettuce by Karam et al. [50] and Zhang et al. [51].

The rise in N availability and the consequent increase in yield also caused an increase in N_{up}, in agreement with the results obtained on several species, including *D. tenuifolia* [52,53]. On the other hand, NUE was higher with the lowest N level, as has already been highlighted for wild rocket [49] and other species [51,54–59]. There are many technicians and researchers who, in order to improve NUE and, at the same time, reduce nitrogen leaching, propose thrifty nitrogen fertilization of vegetables and an adequate water supply [51,60–62].

The application of AZ and SW had no significant effect on the N content of biomass or on N_{up}, even if an increasing trend can be noted (Table 2). The results obtained for AZ disagreed with the literature, in which it is reported that strobilurin fungicides can increase soil N uptake [27,63]. Additionally, for SW, the literature reports an improvement in N_{up}, as observed in wheat seedlings and barley [15,64].

As demonstrated in previous research, most of the examined NUE indicators have highlighted that SW has a positive effect on this important parameter [14,58,59]. These results could be related to the fact that SW modifies root architecture and density, improving the efficiency of mineral uptake [12,14,65]. The increase in NUE could also be explained by the increase in root growth [12], which provided more N uptake capacity [15].

Previous authors have reported an increase in NUE in 'baby leaf' lettuce and spinach treated with seaweed extract, but without data on nitrogen metabolism [66]. The increase in the NUE of lettuce was attributed to phytochemical efficiency and improved activation of photosystem 2 (as demonstrated by the increase in chlorophyll). In spinach, on the other hand, a significant increase in ascorbic acid and polyphenols was observed. The increase in these secondary metabolites has been implicated in key enzyme activities, such as those of the enzyme chalcone isomerase (associated with phytochemical homeostasis through the synthesis of flavanone precursors) [67]. Rouphael et al. [68] also observed increases in polyphenols, ascorbic acid, and the SPAD index in spinach grown under glasshouse conditions using seaweed extracts from *Ecklonia maxima* and *Ascophyllum nodosum* combined with vegetal oils and herbal extracts. Other authors reported that SW application on barley and wheat enhanced N uptake, transport, and assimilation markers at phenotypic, metabolic, enzymatic, and genetic levels. The efficient uptake of nitrate by the crop resulted in enhanced NUE [15,64].

The AZ application increased NUE, probably due to the positive effects of this compound on nitrogen metabolism, cell proliferation, and growth processes [27,32].

Goodig et al. [69,70] reported that strobilurin fungicides improved the NUE of different wheat cultivars compared to untreated plants. Furthermore, Köehle et al. [71] observed that strobilurin stimulated nitrate uptake in hydroponically grown wheat plants. Further, Carucci et al. [72] noticed an increase in the NUE of wheat following the application of AZ, albeit with differences between genotypes.

Jamieson and Semenov [73] claim that N utilization efficiency depends on both N source-to-sink remobilization efficiency and N assimilation efficiency at the sink. The greater amount of N adsorbed is transported in the form of $N\text{-}NO_3$ and $N\text{-}NH_4$ and then assimilated in the leaf [74]. It has been demonstrated that NADH–NR, which catalyzes the first step in $N\text{-}NO_3$ assimilation, is the relevant target for the yield effect of strobilurin [75]. Köehle et al. [71] reported that when wheat plants were treated by strobilurin, nitrite and ammonia were accumulated in the leaf during the night due to the fact that NR was not dark-inactivated as in the untreated plants. Moreover, Glaab and Kaiser [76] reported that strobilurin could cause an additional activation of NR, which is probably mediated via acidification of the cytoplasm.

4.2. Water Use Efficiency

In our research, a moderate water deficit determined the increase in p_WUE, while with a more severe deficit this parameter was not influenced by the irrigation regime, which is in agreement with the literature. In fact, under low water stress, a small reduction in stomatal conductance can have protective effects against stress, allowing the plant to save water and consequently improve WUE [77,78].

Both water shortages and increased N availability promote an increase in p_WUE, which is in agreement with the results of Yin et al. [79] and with the general theory that supplying a limiting resource can improve the use efficiency of other resources [80].

SW determined an increase in p_WUE because of the increase in net assimilation, without significant changes in transpiration rate. The increase in p_WUE occurred mainly in conditions of water deficit, confirming reports relating to the presence of osmotically active components in seaweed extracts [22,81], which can alleviate some abiotic stresses such as drought stress [65,82,83]. In addition, the increase in p_WUE with the application of SW could also originate from the stimulation, by the biostimulant, of the production of ABA [84], a hormone which notoriously intervenes in the regulation of the stomatal opening.

The application of azoxystrobin increased p_WUE more under severe water deficit. This can be attributed to the combined effect of increasing leaf chlorophyll content and improving plant water status, which is determined by the reduction of stomatal conductance [28,35].

The values of Y_WUE, B_WUE, and IY_WUE were significantly lower than those obtained for wild rocket by Cantore et al. [85] and Candido et al. [9] in open-field conditions, which highlights how wild rocket is one of the vegetables with the highest WUE values. One of the factors probably contributing to the high WUE of this species compared to other leafy vegetables is the difference in photosynthetic metabolism. In fact, *D. tenuifolia* is a species with an intermediate carbon cycle (C_3-C_4) that is known for its high photosynthetic efficiency [86], which would cause a higher biomass yield compared to evapotranspirated water.

The lowest WUE values recorded in this trial are probably attributable to the different cultivation conditions, in particular the different plant densities. The low plant density of the trial carried out in pots (about 165 plants m^{-2}), compared to the field conditions described by Candido et al. [9] (over 1.500 plants m^{-2}), would have favored water loss by evaporation from the soil, which notoriously represents the main factor that reduces WUE [47]. For the calculation of WUE, not only is transpiration taken into consideration, as in the case of p_WUE, but also soil evaporation.

Water shortage improved the WUE of wild rocket. There are many experimental findings reported in the literature that agree with our results, demonstrating that plants can increase WUE as a strategy to reduce the negative effects of drought stress [87], thus reaching a compromise between assimilation and transpiration [77,88]. However, in the presence of strong and prolonged drought stress, WUE generally tends to decrease due to the closure of the stomata and the strong reduction in yield.

In fact, the response in terms of WUE to water shortage depends on the level of water stress that the crop experienced during the growing cycle. Under mild water stress conditions, when a slight stomatal closure occurs, transpiration decreases more than photosynthesis, and WUE consequently increases [7,89–91]. Conversely, severe water stress can lead to the closure of stomata, a drastic decrease in assimilation rate, and a concomitant reduction in WUE [77].

The increase in the N level resulted in a considerable rise in WUE. Several studies have shown that WUE increases as N availability improves [51]. However, WUE has been observed to rise up to certain N levels, while decreasing with excessive doses of N [92].

The seaweed extract-based biostimulant and azoxystrobin, as well as causing an increase in photosynthesis without significantly influencing transpiration, favored the rise in WUE. Experimental evidence in this sense was obtained from tomatoes treated with azoxystrobin or pyraclostrobin and subjected to water shortage [7,30,93].

The positive effect of azoxystrobin on WUE can be attributed to the action of this compound in the regulation of stomatal conductance by promoting the biosynthesis of ABA, a hormone which is involved, as it is well known, in the regulation of stomata opening [94].

5. Conclusions

The interactive effect of the water regimes, nitrogen levels, and two biostimulants (azoxystrobin, a fungicide belonging to the strobilurins group, and a biostimulant based on seaweed extracts) was studied to improve knowledge on this topic. This is expected to have a positive impact on wild rocket production companies by helping them to use resources sparingly while reducing production costs and negative impacts on the environment.

The percentage of total N in the AGB and roots increased with increasing doses of N. Furthermore, N uptake increased with increasing N dose and water availability. NUE decreased with water deficits and with increasing N doses. WUE was slightly higher with water deficits, while it increased considerably with increased N doses.

Seaweed extracts and azoxystrobin have confirmed their efficacy as biostimulants for wild rocket. In particular, although to different extents, they both contributed to increasing WUE and some NUE indicators. The biostimulating effect was more evident in conditions of water deficit.

Therefore, it can be stated that the two biostimulants used in this research can represent a valid tool that wild rocket farmers can use to reduce the required input of water and nitrogen without considerable production losses. This would help reduce production costs and the risk of polluting aquifers. We believe that the information acquired in this study can provide valid support for the application of these two biostimulants in the cultivation of wild rocket on the basis of the scientific literature concerning other species.

It would certainly be useful to validate the results obtained in the present study with a similar field trial that also studies aspects related to doses and application timings for the two biostimulants. The results obtained for wild rocket could be extrapolated to other crops, especially those with the same characteristics as other leafy vegetables; however, an experimental verification would be advisable.

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