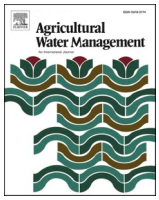
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Interactive effect of water regime, nitrogen rate and biostimulant application on physiological and biochemical traits of wild rocket

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ABSTRACT

A research was carried out to evaluate the biostimulating effects of Azoxystrobin (Azo+) and a brown seaweed extract (SW+) on wild rocket grown during two crop cycles in pots placed in a cold greenhouse. Two watering regimes were applied (restoring 50% and 100% of crop evapotranspiration, indicated respectively WR_{50} and WR_{100}) along with three N levels (0, 75 and 150 kg ha^{-1} of N). The experimental layout was a split-split plot with three replications, arranging water regimes in the main plots, N levels in the plots and biostimulants in the sub-plots. The paper reports the results related to the effects on i) leaf chlorophyll content (Chl), ii) parameters related to gas exchanges including net assimilation (A), transpiration (T), stomatal conductance (gs), internal CO_2 concentration (Ci) and intrinsic water use efficiency (WUEi), iii) some enzymes involved in oxidative stress including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT). The water deficit led to a drop in A and T, an increase in WUEi and in the antioxidant enzymes content of wild rocket. Chl rised with the N availability increase. The increase in N level caused the rise of A and T in WR_{100} while it exacerbated the negative effect of water deficit in WR_{50}. Azo+ caused 15.0% increase in A, 13.4% drop in gs and 33.5% rise in WUEi. Additionally, Azo+ increased by 8.7% Chl and by 489%, 112%, 193%, and 336% SOD, POD, APX and CAT, respectively. SW+ improved A (+7.9%), WUEi (+14.9%), Chl (+6.1%), SOD (+395%), POD (+160%), APX (+155%) and CAT (+334%). The increase in antioxidant enzymes after the application of Azo+ and SW+ was greater under water deficit. The wild rocket benefited of Azo+ and SW+ application, which stimulated the biosynthesis of chlorophyll and antioxidant enzymes, demonstrating the potential role in limiting water stress. Therefore, the two biostimulants can represent a useful tool to improve the production of wild rocket and increase the water use efficiency.

1. Introduction

In Italy, the wild rocket [*Diplotaxis tenuifolia* (L.) DC.] is an expanding leaf vegetable due to its qualitative peculiarities (Cavaiuolo and Ferrante, 2014; Caruso et al., 2019). It is among those vegetables that provides fast economic return since it is a fresh-cut product whose market is constantly growing. The main cultivation method is in open air or under plastic tunnels with a high plant density (1500–2500 m^{-2} plants) to obtain a baby leaf product with harvesting at the stage of about 8 leaves (Schiattone, 2018).

Fresh-cut products require high quality standards that can be achieved by applying appropriate agronomic techniques timely and in the most adequate way. The agronomic factors that have the greatest impact on yield and its quality include water and nutrition management, especially nitrogen (N). As known, water availability is one of the main production factors for all crops and, in particular, for leafy vegetables. Moreover, the management of water and fertility is among the factors that have the greatest impact on the sustainability of production, especially in terms of environmental impact. Water shortage occurs frequently in hot-arid areas, like Southern Italy, which requires a smart management of water and nutrient inputs due to the risks of misuse by farmers and consequent negative impacts on the environment. Crop

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water stress decreases yield of many leafy vegetables as a consequence of the leaf number and size drop, closely linked to the reduction of net assimilation (Bozkurt et al., 2009; Mahmood et al., 2004; Mirdad, 2009). Usually, *D. tenuifolia* grows wildly in poor soils under water shortage conditions. It assumes a xeromorphic habitus that allows the survival and reproduction under water stress, but inadequate to market purposes. Stable production of high yield quality for fresh-cut products is instead obtained by ensuring an adequate water supply (100% of crop evapotranspiration) during the whole growing season (Schiattone et al., 2018b).

Furthermore, wild rocket, like other leafy vegetables, requires an adequate N supply to obtain high yield and excellent quality. In fact, the availability of N influences numerous qualitative parameters of leafy vegetables (i.e. dry matter, chlorophyll, polyphenols, ascorbic acid, nitrates, leaf color and brightness, shelf life, crunchiness (De Pascale et al., 2013).

Considering the economic importance of wild rocket and the essential role of water and nitrogen availability during the growing cycle, it is necessary to pay particular attention to the optimal management of water and nitrogen supply.

Modern agriculture focusses on the optimization of resources-use while decreasing the negative impacts on the environment and increasing quantity and quality of products and, therefore, economic benefits. Meeting these goals could be favoured by using natural products known as plant biostimulants (Colla and Rouphael, 2015; Baltazar et al., 2021; Li et al., 2022). 'Plant biostimulants contain substance(s) and/or microorganisms whose function, when applied to plants or to rhizosphere, is to stimulate natural processes that enhance nutrient uptake, efficiency, tolerance to abiotic stress, and crop quality, with no direct action on pests' (EBIC, 2013).

Among the numerous substances and organisms for which some biostimulating action has been proven, du Jardin (2015) reported the seaweed extract whose composition and bioactive components are not fully known. Furthermore, numerous experimental findings showed a biostimulating action also by strobilurins, compounds of natural origin commonly used as broad-spectrum fungicides.

The promotion of plant growth by some seaweeds has been known for some time, so that their use as organic fertilizers has become widespread (Craigie, 2011). But, since the 1950 s, the use of whole seaweed has been progressively replaced by extracts of different species of seaweeds (du Jardin, 2015). Seaweed extracts contain inorganic and organic substances in varying quantities depending mainly on the seaweed species and the extraction method. Among the inorganic substances there are N, P, K, Ca, Fe, Mg, Zn, Na and S. Organic compounds include amino acids, osmolytes (e.g. mannitol, betaines and betaine analogues), secondary bioactive metabolites, vitamins and vitamin precursors, polysaccharides, phenols, phytohormones (e.g. auxins, cytokinins, gibberellins, abscisic acid, acid acetic indole, polyamines and brassinosteroids) (Boukhari, 2020). Therefore, the biological activity of the extracts can differ considerably in relation to the starting raw material and the extraction process (Khairy and El-Shafay, 2013; Kim, 2012; Puglisi et al., 2020). However, it is difficult to interpret the results obtained following of the use of these products and the attribution of any effects to one or the other of the components present in the extract (Boukhari et al., 2020).

There are numerous experimental findings that demonstrate a complementary biostimulating action of strobiluins at a metabolic and physiological level on different species, which often leads to an improvement in yield and quality (Amaro et al., 2020; Boari et al., 2019; Candido et al., 2020; Schiattone et al., 2021). Strobilurins are substances of natural origin from which various structural variants have been synthesized (e.g.: Azoxystrobin, Kresoxim-methyl, Pyraclostrobin). They are used in agriculture as fungicides and their spreading has been favored by their low toxicity to humans and the environment. In nature these substances are produced by some Basidiomycetes fungi [e.g.: *Oudemansiella mucida* (Schrad ex Fr) Hoehn and *Strobilurus tenacellus* (Pers ex Fr) Singer] which cause the wood rot of some tree species (Balba, 2007; Bartlett et al., 2002).

In addition to the fungicidal action, the application of strobilurins seems to lead to the increase in abscisic acid (ABA) and some enzymes involved in oxidative stress as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) (Boari et al., 2019; Liang et al., 2018; Lopes et al., 2018) which can, therefore, positively influence assimilation rate, transpiration, stomatal conductance and water use efficiency (WUE) (Cantore et al., 2016; Giuliani et al., 2018). Furthermore, a positive action has been demonstrated on cell proliferation (van Dingenen et al., 2017), N metabolism and N-NO_3 accumulation, probably due to the stimulating action of nitrate reductase activity (Amaro et al., 2018, 2020; Joshi et al., 2014), as observed on lettuce and spinach treated with Azoxystrobin (Bonasia et al., 2013), on which an increase in chlorophyll, vitamin C and phenolic compounds was observed (Conversa et al., 2014).

In literature, there are little experimental findings on the effects of biostimulants such as strobilurins and seaweed extracts on wild rocket. Therefore, a study was carried out to verify the combined effect of water regime, nitrogen level and two biostimulants (Azoxystrobin and seaweed extract) on the performance of wild rocket during two growing cycles. In particular, this study reports the effects on some physiological parameters (related to gas exchange) and biochemical aspects (chlorophyll content and some antioxidant enzymes) strictly connected to the agronomic variables. This knowledge can be of great use to optimize the application of resources and to improve the production performance of wild rocket while reducing the environmental impact of its cultivation.

2. Material and methods

2.1. Characteristics of the experimental site

The research was carried out at the Mediterranean Agronomic Institute (CIHEAM) of Bari (Italy) (41°03′ N, 16°52′ E; 72 m a.s.l.). During the autumn-winter 2016–2017 (15 November – 17 Genuary and 18 Genuary - 28 February) two crop cycles were carried out in an unheated greenhouse, covered with ethylene vinyl acetate (EVA) film (200 μm in thickness). This area is characterized by a typical Mediterranean climate.

The experiment was carried out using cylindrical pots (0.34 m in diameter and 0.3 m in height) suitably equipped with saucers, each containing 20 dm^3 of soil (type Lithic-Ruptic-Inceptic-Haploxeralfs; USDA, 2006) of good fertility taken in the same locality. The soil of the most superficial layer (0–5 cm) was mixed with perlite (soil/perlite ratio 5:1, v/v), to prevent compactation by irrigation water.

2.2. Climatic trend

Two growing cycles of wild rocket were characterized by daily minimum (T_{min}) and maximum (T_{max}) temperatures which fluctuated, respectively, between − 2.1 and 16.9 °C and between 1.4 and 23.5 °C. In particular, during the first crop cycle, in the first decade of January, very low temperatures occurred, with values of T_{min} for several days below 0 °C and T_{max} that in the same days did not exceed 5.5 °C. During the second crop cycle T_{max} increased progressively until harvest, oscillating around 16 °C, while T_{min} remained around 7 °C (data not shown).

The solar global radiation (Rg) showed a steady trend, around of 7.5 MJ m^{-2} d^{-1}, from transplantation until the end of January. Subsequently, the trend of this parameter has been increasing up to about 14 MJ m^{-2} d^{-1} in the days preceding the last collection (data not shown).

2.3. Treatments, experimental layout and crop management

The following three experimental treatments were compared: two watering regimes (W*R*) (restoring 50% and 100% of crop evapotranspiration-ETc, indicated respectively WR_{50} and WR_{100}); three

N levels (NL) (0, 75 and 150 kg ha^{-1} N, indicated respectively N_0, N_{75} and N_{150}); the application of two Biostimulants (BS) based on Azoxystrobin (Ortiva®, Syngenta) and seaweed extract (Bioproject SM23 Foliar-BioKimia® International Srl), indicated respectively Azo+ and SW+ , in addition to a untreated control (C). The experimental layout was a split-split plot with three replications, arranging W*R* in the main plots, NL in the plots and BS in the sub-plots each consisting of one pot.

Fertilization was carried out as follows: 3.34 and 1.25 g pot^{-1} of P_2O_5 (19% superphosphate) and K_2O (51% potassium sulphate), corresponding respectively to 70 kg ha^{-1} of both nutrients, distributed before transplantation in all treatments and then buried at a depth of 5–10 cm. Furthermore, treatments N_{75} and N_{150} were fertilized, respectively, with 1.73 and 3.46 g pot^{-1} of ammonium sulphate 21% (40 and 80 kg ha^{-1} of N) before transplantation and with 1.51 and 3.02 g pot^{-1} of the same fertilizer (35 and 70 kg ha^{-1} of N) just after the first harvest. Weeds were manually eliminated a few days after emergence to avoid competition with wild rocket plants.

The transplant of wild rocket was performed on November 15th, 2016 using seedlings at the 5th true leaf stage, prepared in the nursery in 228-hole polystyrene alveolate trays. In each pot 5 tufts of 3 seedlings each have been transplanted, suitably spaced. Ortiva® (1.5 mL L^{-1}) and Bioproject SM23 Foliar (1.5 mL L^{-1}) were sprayed 20, 50, 84, and 94 days after transplant (DAT).

For the pest and disease control, only one insecticide (Confidor® 200 SL - Bayer CropScience S.r.l. - Italy., containing Imidacloprid 17,1%) application was necessary to fight an initial attack of aphids (*Myzus persicae*) during the first crop cycle.

Evapotranspiration (ET) was measured by the water balance method by weighing the pots (every 4–5 days) which were considered to be weighing lysimeters (Schiattone et al., 2017).

To meet the water needs of the wild rocket, the water, having an electrical conductivity of 0.5 dS m^{-1}, was manually distributed on the surface of the pots. Watering was carried out when 45% of the available water (AW) ran out in the WR_{100} treatment, based on the threshold value (p = 45) of other Brassicaceae (Allen et al., 1998). Any percolation water was collected in the saucers, which was subsequently weighed and used for the subsequent watering of the same pot, in order to recover any loss of nutrients due to leaching.

Two crop cycles were carried out with harvests made on January 17th (63 DAT) and February 28th (105 DAT).

3. Leaf chlorophyll content

In some days (29, 38, 45, 51, 60, 86, 90, 94, 98 and 105 DAT) during the two growing cycles, the leaf chlorophyll (Chl) content was measured by a non-destructive method, using a chlorophyll meter SPAD-502 (Minolta, Ramsey, NJ), which provides the Chl content in arbitrary units (SPAD units). For each pot, the measurements were performed on three young fully expanded leaves randomly selected for each of five plants, attributing to each pot the average value of fifteen leaves.

4. Gas exchange

Gas exchanges (CO_2 assimilation rate - A, transpiration - T, stomatal conductance - gs, internal CO_2 concentration - Ci) were measured using a portable Li-6400 open system photosynthesis meter (LI-COR, Lincoln, Nebraska, USA), with the light intensity of the leaf chamber (PARin), provided by a red light diode source on the upper surface of the leaf, set at 1800 μmol photon m^{-2} s^{-1}, and the concentration of CO_2, provided by a reservoir of external CO_2, set at 400 μmol mol^{-1}. Intrinsic water use efficiency (WUEi) was calculated as A/gs ratio.

Measurements were made from 11:00–13:30 of two days (61 and 104 DAT) differing mainly in air temperature, vapor pressure deficit (VPD), and soil water deficit (in WR_{50} treatment). On each measurement date, two apical leaves of two plants per pot were chosen, fully expanded and well exposed to light. The order followed for the measures of different treatments was completely random. Each measurement lasted about 2.5 min

The measurements were performed under the following mean climatic conditions in the greenhouse: PAR 861 μmol m^{-2} s^{-1} (61 DAT) and 965 μmol m^{-2} s^{-1} (104 DAT); air temperature 12.4 °C (61 DAT) and 22.5 °C (104 DAT); vapor pressure deficit (VPD) 1.5 (61 DAT) and 2.8 kPa (104 DAT). Furthermore, in the same days, the average water depletion (WD) in the soil was the following: WD equal to about 20% of AW for WR_{100}, 50% (61 DAT) and 90% (104 DAT) of AW for WR_{50}.

4.1. Antioxidant enzyme assay and protein determination

At each harvest time, the enzymatic activity of superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT), was determined. A homogeneous sample of mature and healthy leaves was taken from each pot, immediately closed in a black plastic bag and placed in a portable refrigerator at a temperature of 4 °C. The samples were then transported to the laboratory and analysed within two hours. For the enzymes extraction, 1 g of plant material was randomly taken from the main sample and homogenized in a cold mortar with 1 mL of specific buffer. For SOD: 50 mM K-phosphate (pH 7), 1% Triton X-100, 7 mM 2-mercaptoethanol; for POD: 100 mM Na-phosphate (pH 7.3); for APX: 100 mM Na-phosphate (pH 7) containing 5 mM ascorbate and 1 mM EDTA; for CAT: 50 mM K-phosphate (pH 7), 1% PVP. The homogenates were centrifuged (14,000 *g*, 10 min, 4 °C) and the resultant supernatants were collected to evaluate the enzyme activities. For each enzyme three replicates were performed. The enzymatic activities of SOD, POD, APX and CAT were assessed as previously described by Sergio et al. (2012).

Protein content was measured according to Bradford (1976), using BSA as a standard.

5. Statistical analysis

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

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

6. Results

6.1. Leaf chlorophyll content

The chlorophyll content determined with the SPAD did not show significant variations between the irrigation regimes during the trial, while it differed among N levels and the biostimulant treatments (Fig. 1). In general, the trend of the SPAD index was increasing during each growing cycle, with values that, on average, went from about 35 in mid-December to about 50 at the first harvest, and from 42 in the first ten days of February to about 49 to the second harvest (Fig. 1).

In terms of NL, N_1 and N_2 presented values of 14.3% and 20.5% higher than N_0 (Fig. 1b), respectively.

SW+ and Azo+ resulted in an increase in the SPAD index of 6.1% and 9.1% respectively (Fig. 1c). In particular, for SW+ the increase was more significant in January in the period preceding the harvest, while during the second crop cycle the differences, in respect to the control, gradually decreased until they disappeared near the harvest. Also in the case of Azo+ , the most significant differences with respect to the control were detected in 15 days preceding the first harvest.

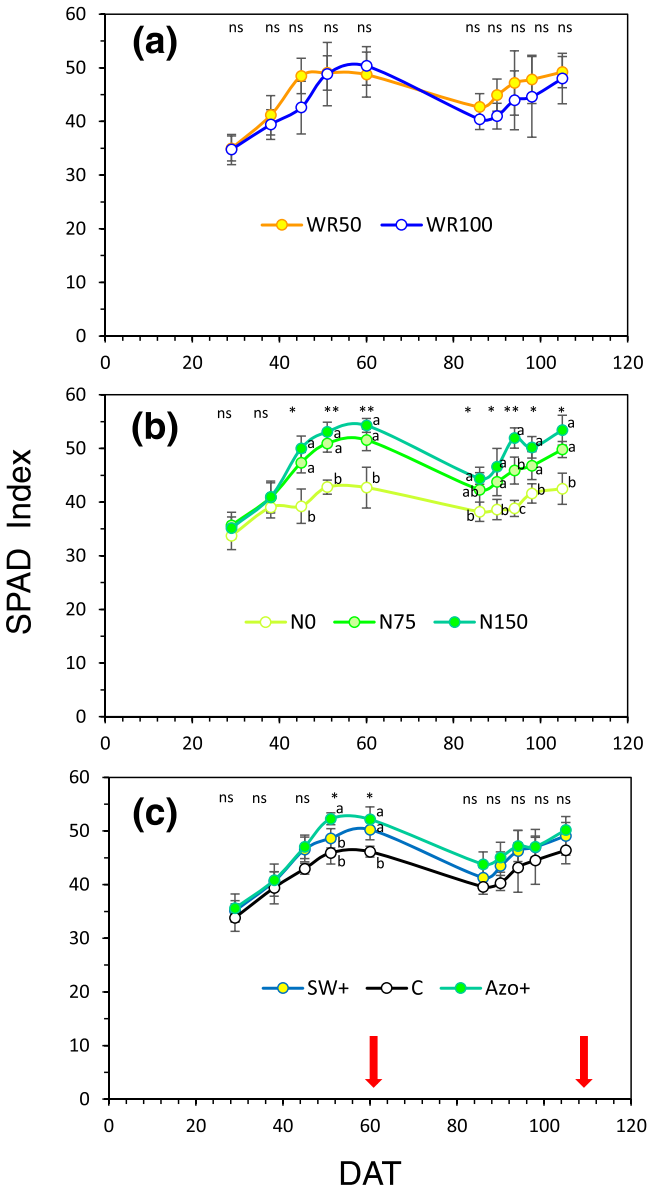


Fig. 1. Effect of water regime (a), nitrogen level (b) and application of biostimulants (c) on the SPAD index during the growing cycle of wild rocket. Arrows indicate harvests; ns, * , * * indicate non-significant or significant F-test at $P < 0.05$ and $P < 0.01$, respectively. Different letters within each measurement date indicate significantly different values according to the SNK test ($P = 0.05$). Vertical bars indicate standard deviation. WR_{100} = restoring 100% of the ETc, WR_{50} = restoring 50% of the 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, Azo+ = application of Azoxystrobin, SW+ = application of seaweed extract based biostimulant.

6.2. Gas exchange

The gas exchanges of the wild rocket were measured in two representative days (61 DAT and 104 DAT) which differed in the soil water deficit level. In WR_{100} on both days, the water reserve was within the readily available water corresponding to deficit of about 20% of total available water (TAW). However, in WR_{50}, water deficit was equal to 50% and 90% of TAW on 61 DAT and 104 DAT, respectively. Overall, different water deficit levels observed for the treatments influenced the parameters describing the gas exchange of wild rocket (Table 1). The lower water availability of WR_{50} in respect to WR_{100}, caused a reduction of A, T and gs by 11.1 – 25.6% and 23.9%, and by 51.8 – 52.5% and 55.6%, respectively on 61 and 104 DAT, which corresponded to a decrease in Ci, above all on 104 DAT (Table 1).

The A increased by 23.3% and 10.5% from N_0 to N_{75}, on 61 and 104 DAT respectively, while no further increase was found with the highest NL.

Transpiration rised by 11.4% from N_0 to N_{75} on both dates of measurement. A further increase of nitrogen dose (N_{150}) caused a slight increase of A on 61 DAT, while on 104 DAT this parameter was reduced by 6.8%.

The gs trend in relation to the NL was opposite for two dates of measurement. The gs rised with the increase of NL on 61 DAT, whereas it was reduced on 104 DAT. The Ci did not show variations in relation to NL on 61 DAT, while it decreased with the increase of NL on 104 DAT.

The increase of NL exacerbated the negative effect of the water deficit on A and T (*WR* x NL interaction) (Fig. 2), especially on 104 DAT, when water-deficient plants have experienced longer and more severe water stress.

Azo+ and SW+ , on average, increased A by 15.0% and 7.9%, respectively. Furthermore, Azo+ reduced T by 10.6% on 104 DAT and gs by 20.0% and 6.7% on 61 and 104 DAT, respectively.

The values of WUEi varied significantly in relation to the compared treatments (Table 1, Fig. 3). When in WR_{50} the water deficit was lower (WD = 50% of AW, on 61 DAT), a 16.9% increase in WUEi was recorded compared to WR_{100}, while under more severe water stress (WD = 90% of AW, on 104 DAT) the positive effect of the water deficit on this parameter was smaller (Table 1). In addition, the WUEi increased progressively with the increase of NL.

The Azo+ and SW+ resulted in an average increase of 15.0% and 33.1% in WUEi, respectively. The increase of the latter parameter after the sprying of SW+ occurred mainly in conditions of water deficit (*WR* x BS interaction) (Fig. 3).

6.3. Antioxidant enzymes (SOD, POD, APX, CAT)

Four examined enzymes were affected to a different extent by the treatments (Fig. 4). The enzymes were significantly affected by different wate regimes. In particular, their concentration was higher in WR_{50} compared to WR_{100} by 391.7 (SOD), 374.2 (POD), 42.1 (APX) and 137.1% (CAT), as the average of two harvests.

The NL did not affect any of studied enzymes.

SW+ and Azo+ significantly stimulated the production of SOD, POD, APX and CAT whose values, as the average of two harvests, were respectively 395.4%, 160.5%, 155.0% and 333.9% and 489.5, 112.4, 192,9% and 335.9% higher than the control.

The differences between Azo+ , SW+ and control treatments were significantly more pronounced in conditions of water deficit than under optimal water supply (*WR* x BS interaction) (Fig. 5).

7. Discussion

7.1. Leaf chlorophyll content

Chlorophyll content of wild rocket increased by increasing N level and applying Azoxystrobin or seaweed extract based biostimulants.

The increase in chlorophyll in relation to the rise in NL is consistent with the results obtained on wild rocket (Candido et al., 2020; Schiattone et al., 2018b), lettuce (Bonasia et al., 2013) and spinach (Conversa et al., 2014), as it would expect, since nitrogen is one of the main constituents of the pigment (Taiz and Zeiger, 2002).

The chlorophyll content of wild rocket leaves increased following the application of seaweed extract. Similarly, Puglisi et al. (2020) reported a considerable increase in chlorophyll in lettuce leaves with the application of extracts of microalgae (*Scenedesmus quadricauda*), just as the increase of this pigment has also been observed in corn leaves treated with extracts of different species of algae (Fayzi et al., 2020). The increase in chlorophyll following the application of the seaweed extract can be attributed to increased availability of algal nutrients, especially

Table 1
Effect of water regime (WR), nitrogen level (NL) and application of biostimulants (BS) on the parameters relating to the gas exchange of wild rocket 61 (January 15) and 104 (February 27) days after transplantation (DAT). A = Net assimilation; T = transpiration; gs = stomatal conductance; Ci = internal CO_2 concentration; WUEi = intrinsic water use efficiency (A/gs). WR_{100} = restoring of 100% of ETc, WR_{50} = restoring 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, Azo+ = application of Azoxystrobin, SW+ = application of seaweed extract based biostimulant.

Treatments	A ($\mu mol\ m^2\ s^{-1}$)	T ($mmol\ m^2\ s^{-1}$)	gs ($mol\ m^2\ s^{-1}$)	Ci ($\mu mol\ CO_2\ mol^{-1}$)	WUEi ($\mu mol\ CO_2\ mol^{-1}\ H_2O$)
61 DAT					
Water Regime (WR)	* *	* *	* *	ns	*
WR_{50}	19.3	3.2	0.35	232.4	55.1
WR_{100}	21.7	4.3	0.46	244.1	47.2
Nitrogen Level (NL)	* *	*	ns	ns	*
N_0	17.6 b	3.5 b	0.39	240.4	45.1 b
N_{75}	21.7 a	3.9 a	0.41	236.7	52.9 ab
N_{150}	22.3 a	4.0 a	0.40	237.9	55.8 a
Biostimulants (BS)	*	ns	*	ns	*
C	19.4 b	3.9	0.45 a	238.9	43.1c
Azo+	21.5 a	3.7	0.36 b	237.6	59.7 a
SW+	20.5 a	3.7	0.41 ab	238.4	50.0 b
WR x NL	*	*	ns	ns	ns
WR x BS	ns	ns	ns	ns	*
NL x BS	ns	ns	ns	ns	ns
WR x NL x BS	ns	ns	ns	ns	ns
104 DAT					
Water Regime (WR)	* *	* *	* *	* *	*
WR_{50}	11.9	2.9	0.18	155.0	66.1
WR_{100}	24.7	6.1	0.41	201.3	61.0
Nitrogen Level (NL)	*	*	*	* *	*
N_0	17.1 b	4.4 ab	0.30 a	212.3 a	57.0 b
N_{75}	18.9 a	4.9 a	0.29 ab	166.6 b	65.1 ab
N_{150}	19.0 a	4.1 b	0.28 b	156.7c	67.9 a
Biostimulants (BS)	*	*	*	*	* *
C	16.7c	4.7 a	0.30 a	185.3 a	55.7c
Azo+	19.9 a	4.2 b	0.28 b	174.8 b	71.1 a
SW+	18.4 b	4.5 ab	0.29 ab	175.3 b	63.4 b
WR x NL	* *	* *	ns	ns	ns
WR x BS	ns	ns	ns	ns	*
NL x BS	ns	ns	ns	ns	ns
WR x NL x BS	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 ($P = 0.05$).

nitrogen and magnesium, which are fundamental components of chlorophyll forming (Rahmawati et al., 2015). Furthermore, the increase of chlorophyll content might also be explained by the decrease in degradation of this molecule, caused in part by cytokinins and betaines, known to be present in seaweed extract (Jannin et al., 2013; Balakumbahan and Kavitha, 2019).

Our study showed that Azoxystrobin significantly increased the leaf chlorophyll content of wild rocket, confirming the data of the literature, in which it is reported that strobilurin favors the increase of chlorophyll during plant development (Bonasia et al., 2013; Liang et al., 2018). This is attributable to its effect on the balance of some phytohormones (Grossmann and Retzlaff, 1997).

In particular, strobilurin would reduce the synthesis of ethylene, a hormone notoriously involved in the processes of senescence and degradation of chlorophyll (Abeles et al., 1992); on the other hand it would favor the increase of cytokinins, involved in the biosynthesis of chlorophyll in the presence of light (Sundqvist et al., 1980).

7.2. Gas exchange

The compared treatments influenced the gas exchange parameters of wild rocket. In particular, the reduction in water availability reduced A, T and gs, which was accompanied by a decrease in Ci, especially when water stress was severe. These results are in agreement with the literature where is widely reported that photosynthesis is among the first physiological processes negatively influenced by the reduction of water availability (Chaves, 1991). It is well documented that under moderate water stress the reduction of A and gs is accompanied by a corresponding decrease in Ci. On the other hand, under strong water stress the reduction of A and gs correspond to relatively higher values of Ci (Wu et al., 2008). This indicates that with moderate water stress, gs is the main limiting factor of assimilation, regardless of any metabolic alteration (Chaves and Oliveira, 2004). With strong water stress, however, the reduction in the mesophyll CO_2 availability caused by the closure of the stomata is not the main cause of the reduction in A, but the reduction in the efficiency of carboxylation is involved (Rouhi et al., 2007). Hence, in our case, it can be deduced that for the wild rocket under water deficit, at least in two measurement days (61 and 104 DAT), the stomatal limitation of photosynthesis prevailed.

The A increased from N_0 to N_{75}, while no further increase was found with the higher NL. Transpiration did not change on the first measurement date, when the water stress in WR_{50} was lower, while on the second date this parameter decreased with the highest NL.

The trend of gs in relation to the NL was opposite in the two measurement dates. In particular, this parameter has shown the tendency to increase with the rise of NL (61 DAT) and then to diminish (104 DAT). On 61 DAT, the Ci did not show variations in relation to the NL, while on 104 DAT it decreased with the increase in NL.

The increase in NL accentuated the negative effect of water limitation on A and T, especially when the water stress, to which the WR_{50} treatment plants were subjected, persisted for longer period and when it was more severe. Also Wu et al. (2008) reported that the response to water deficit of the parameters relating to gas exchanges is different in relation to N availability. These authors observed that under optimal water supply A and gs were higher with high N levels. On the other hand, in conditions of water deficit, the A of plants subjected to a high N intake

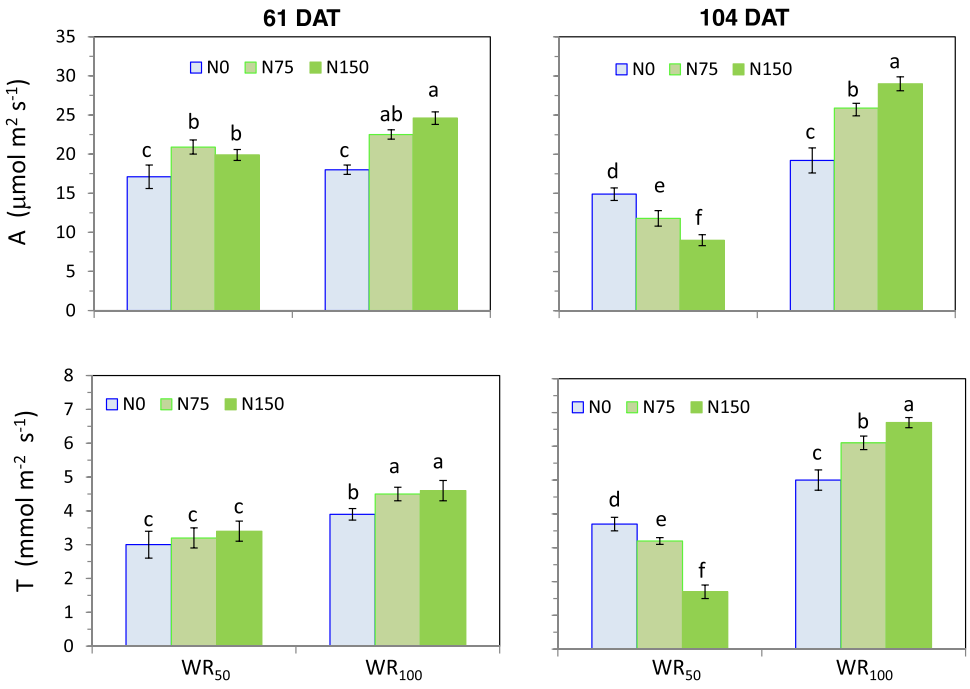


Fig. 2. Water regime x nitrogen level interaction on the net assimilation (A) and transpiration (T) of wild rocket 61 and 104 DAT. Different letters indicate significantly different values according to the SNK test (P = 0.05). Vertical bar indicate standard deviation. WR_{100} = restoring 100% of the ETc, WR_{50} = restoring 50% of the 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.

was inhibited, while no significant N effects on gs were observed.

The SW+ increased A (Schiattone et al., 2018a). This can be associated with the increase of leaf chlorophyll content as obtained in our research, which confirms the findings of other authors (Fan et al., 2013; Jannin et al., 2013).

Azo+ resulted in the increase of A, which can be attributed to the combined effect of the rise in the leaf chlorophyll content and the improvement of the water status of the plant, determined by the reduction of gs. With regard to the gs control and, therefore, of the plant water status, this aspect was not very evident in our study. It might be explained by better physiological conditions of the plants treated with Azoxystrobin which presented a greater stomatal opening. Experimental evidence in this sense, was obtained on tomatoes treated with Azoxystrobin (Giuliani et al., 2011) or Pyraclostrobin (Boari et al., 2019; Cantore et al., 2016). In fact, these authors observed that the application of strobilurin-based products improved the plant water status and gas exchange. These considerations are also supported by the findings of other authors who argue that strobilurins positively influence the endogenous concentration of ABA, which would help inhibit stomatal opening, improving the water use, especially when the plant is in water deficit conditions (Venancio et al., 2003). In addition, Grossmann and Retzlaff (1997) stated that strobilurins causes decreased respiration and, consequently, the change of the CO_2 compensation point and CO_2 net assimilation increase.

A limited water deficit led to an increase in WUEi, while with a more severe water deficit this effect was lower. In fact, in the presence of low water stress, a small reduction in gs can have protective effects against stress, allowing the plant to save water and, consequently, to improve the water use efficiency (Chaves et al., 2009).

The WUEi increased progressively with the increase in NL. Many studies have well documented the photosynthetic response of the plant to the N availability and, some of these, highlighted the existence of a positive correlation between the photosynthetic capacity and the leaf nitrogen content, suggesting that most of the nitrogen is used for the synthesis of compounds of the photosynthetic apparatus (DaMatta et al., 2002; Makoto and Koike, 2007) such as chlorophyll (Maheswari et al., 2008). Both the lack of water and the increase in the N availability promote an increase in WUEi, in accordance with the results of Yin et al.

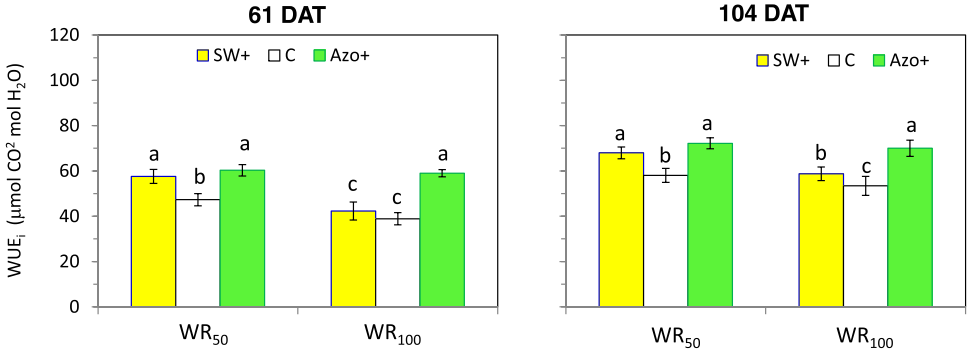


Fig. 3. Water regime interaction x treatments with biostimulants on the intrinsic water use efficiency (WUEi) of the wild rocket 61 and 104 DAT. Different letters indicate significantly different values according to the SNK test (P = 0.05). Vertical bars indicate standard deviation. WR_{100} = restoring 100% of the ETc, WR_{50} = restoring 50% of the ETc. C = control, Azo+ = application of Azoxystrobin, SW+ = application of seaweed extract based biostimulant.

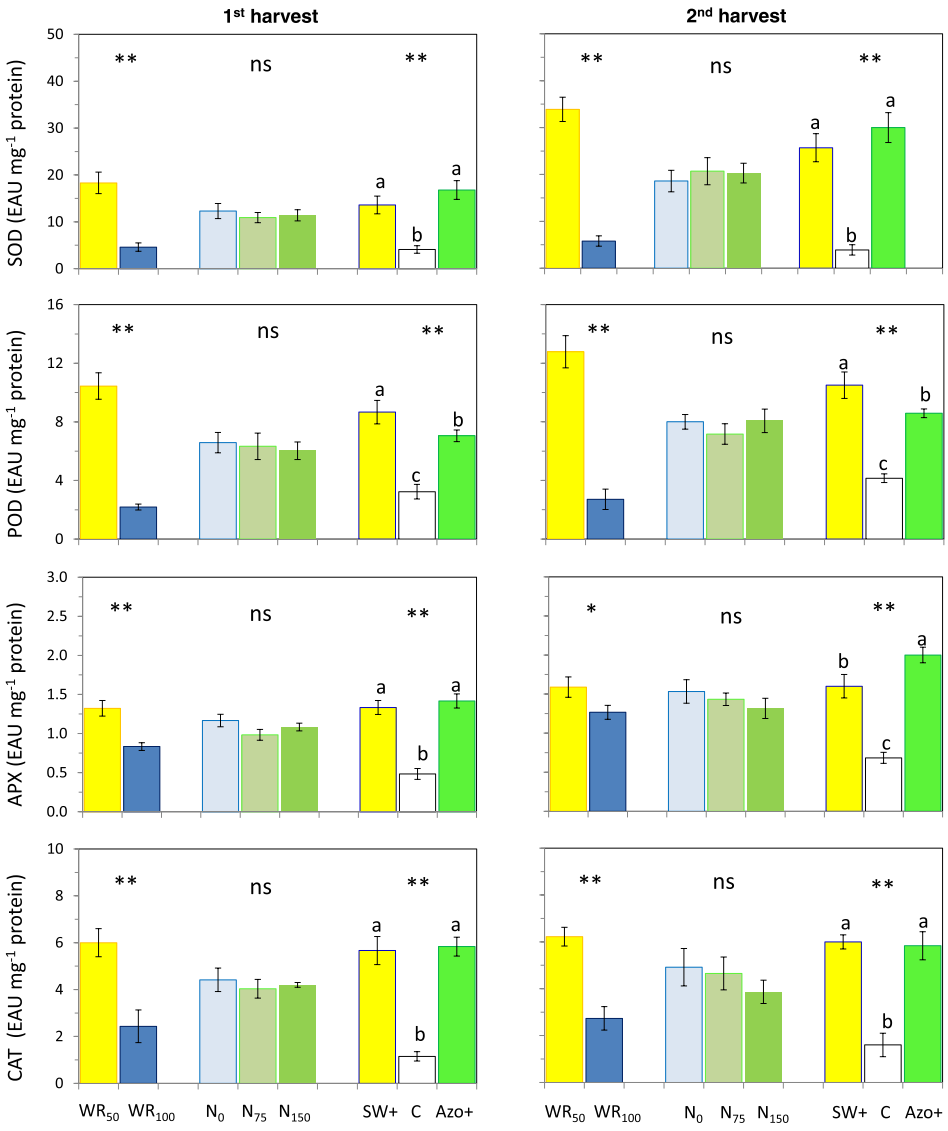


Fig. 4. Effect of water regime, nitrogen level and application of biostimulants on the superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) of wild rocket in the two harvests. EAU = enzyme activity unit. ns, * , * * indicate non-significant or significant F-test at $P < 0.05$ and $P < 0.01$, respectively. Different letters within each treatment indicate significantly different values according to the SNK test ($P = 0.05$). Vertical bars indicate standard deviation. WR_{100} = restoring 100% of the ETc, WR_{50} = restoring 50% of the 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, Azo+ = application of Azoxystrobin, SW+ = application of seaweed extract based biostimulant.

(2006) and with the general theory that the limiting supply of a resource can improve the efficiency of use of other resources (Wu et al., 2008).

The SW+ increased WUEi as a consequence of the increase in A, without significant changes in T. The increase in WUEi after application of SW+ occurred mainly in conditions of water limitations, confirming what is reported in the literature on the presence of osmotically active components in algae extract (Blunden et al., 2009; MacKinnon et al., 2010), which can alleviate some abiotic stress such as water stress (Battacharyya et al., 2015; Neily et al., 2008).

The Azo+ increased WUEi. This can be attributed to the combined effect of the increase in the leaf chlorophyll content and the improvement of the plant water status, determined by the reduction of gs (Bonasia et al., 2013; Liang et al., 2018).

7.3. Antioxidant enzymes (SOD, POD, APX, CAT)

The water deficit caused a significant increase in the enzymes involved in oxidative stress that were examined (SOD, POD, APX and CAT). These results agree with the literature in which the enzymatic response of plant to different biotic and abiotic stress is widely reported, including water stress (Abd El Baky et al., 2016; Sergio et al., 2012; Sofo et al., 2005).

In fact, plants subject to water deficit undergo excessive reducing power due to the reduction of CO_2 assimilation which causes the increase in the levels of activated oxygen species (ROS) and the accumulation of free radicals. If the accumulation of ROS exceeds the ability of the antioxidant system to remove them, then the peroxidation of membrane lipids arises, the destruction of photosynthetic pigments, the inactivation of the enzymes involved in the reactions of CO_2 fixation and, finally, cell death (Møller, 2001; Sairam and Tyagi, 2004). The enzymatic defense system, to eliminate ROS adopted by plants, includes SODs, which catalyze the conversion of superoxide into H_2O_2, together with CAT, PODs and APX, which eliminate the H_2O_2 produced (Sofo et al., 2005; Zhang et al., 2015).

The application of the seaweed-based biostimulant resulted in a significant increase in four enzymes examined in both WR. However, to a greater extent in the presence of water stress, the beneficial effects of seaweed exstracts on water stressed plants through the control of antioxidant enzymes are reported (Hasanuzzaman et al., 2021). This result agrees with the observations of other authors on different species such as potato, wheat and soybean treated with seaweed extract and subjected to water shortage (Abd El-Baky et al., 2008; Abd El Baky et al., 2016; Shukla et al., 2018).

Azoxystrobin also stimulated the production of antioxidant enzymes whose concentration in the aerial part of the wild rocket was significantly higher than in the control. In the literature, experimental findings

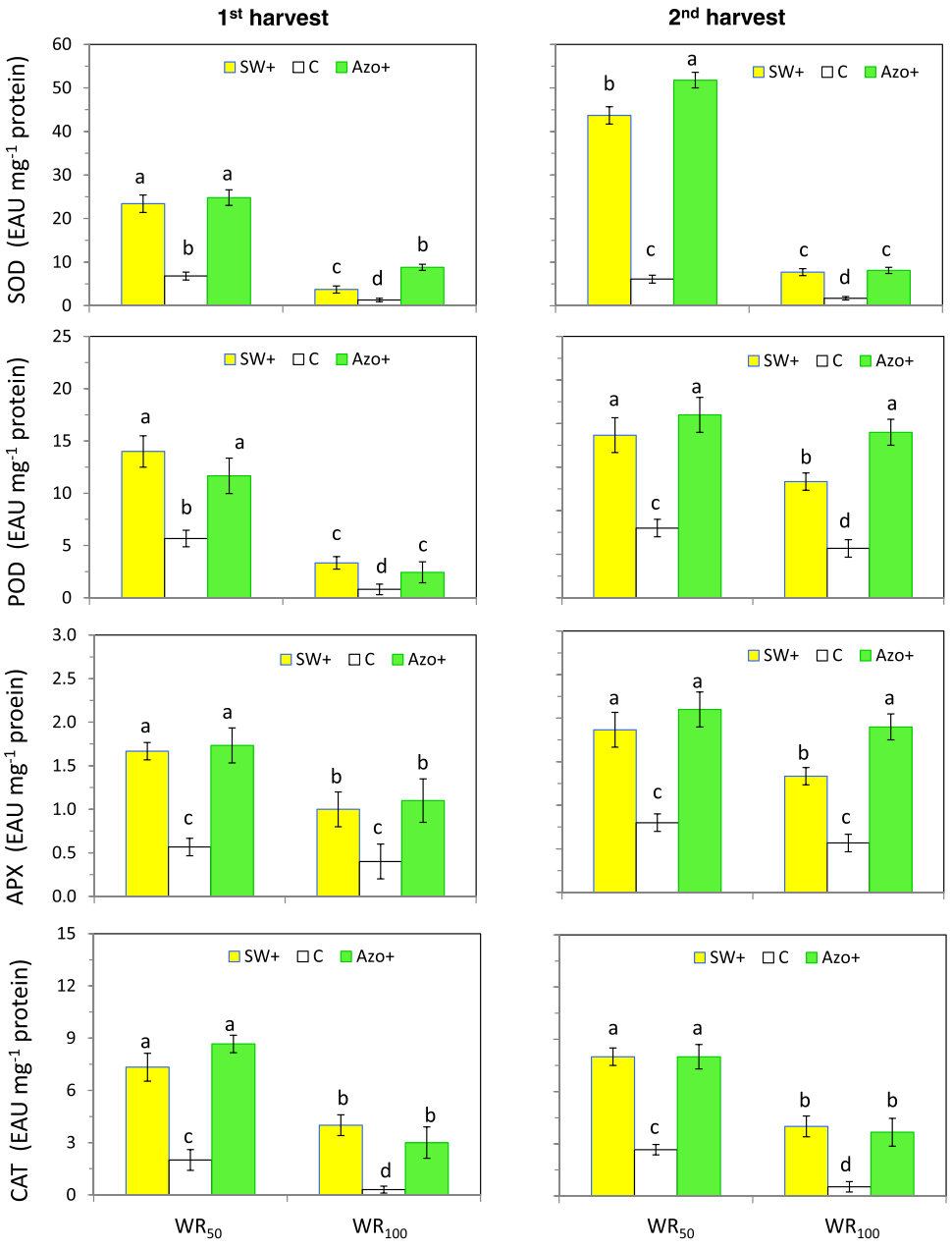


Fig. 5. Water regime interaction x treatments with biostimulants on superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT) of wild rocket in the two collections. Different letters indicate significantly different values according to the SNK test (P = 0.05). Vertical bars indicate standard deviation. EAU = enzyme activity unit. WR_{100} = restoring 100% of the ETc, WR_{50} = restoring 50% of the ETc; C = control, Azo+ = application of Azoxystrobin, SW+ = application of seaweed extract based biostimulant.

in this sense were reported on barley crops, where the application of Pyraclostrobin would have inhibited the manifestation of the 'physiological leaf spot', a physiopathy attributable to oxidative stress; the response of barley to Pyraclostrobin has been attributed to the doubling of the enzymatic activity of peroxidase (Köehle et al., 2003). Cantore et al. (2015) reported an increase in SOD, POD, APX and CAT in tomato plants treated with Pyraclostrobin, which would have improved salinity tolerance, while Liang et al. (2018) found a significant increase in the activity of the main antioxidant enzymes following the application of Azoxystrobin on ginseng, which would have favored the delay of leaf senescence.

8. Conclusions

The interactive effect of irrigation regimes, nitrogen level and the application of Azoxystrobin (fungicide belonging to the strobilurin class) and Bioproject SM23 Foliar (biostimulant based on seaweed extracts) was studied, in order to acquire usefull informations for improving some agricultural techniques (irrigation and nitrogen management, biostimulant application) able to ensure economically viable production levels and high quality characteristics of wild rocket, and to increase water use efficiency.

Both Azoxystrobin and the brown seaweed extract based biostimulant have proven effective in improving some physiological and biochemical parameters of wild rocket, especially in conditions of severe and prolonged water deficit. In particular, the increase in the leaf chlorophyll content, net assimilation and intrinsic water use efficiency was observed. In addition, both substances have stimulated the biosynthesis of the antioxidant enzymes (SOD, POD, APX and CAT), demonstrating the potential role in limiting water stress. The information gained through the research provides useful indications for improving the production techniques of wild rocket and optimizing the use of resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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