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Integrating of Biostimulants Alongside Various Advanced Nitrogen Fertilization Practices Improve the Yield, Quality, and Sustainability of Malting Barley in Mediterranean Conditions

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Abstract

Barley (*Hordeum vulgare* L.) is a key cereal crop for malting and brewing, where grain plumpness and optimal grain protein concentration (GPC) are essential quality traits. This study investigated the combined effects of nitrogen fertilization strategies and a seaweed-based biostimulant (*Ascophyllum nodosum* extract) on malting barley production across four environments in Thessaly, Greece, over two growing seasons. Treatments included urea (U), urea with biostimulant (U+B), urea with urease inhibitor (UI), urea with urease inhibitor and biostimulant (UI+B), and a control (no fertilization). Applications were tested on genotype G20 at mid-development (Z30–33) and genotype G45 at an earlier stage (Z24–30). UI+B treatment consistently enhanced yield by up to 71%, thousand grain weight by 27%, and spikelets per square meter by 75% relative to the control, with responses influenced by genotype and environment. Grain fractions >2.8 mm increased by up to 22% under UI+B, while GPC remained within the optimal malting range (9.5–11.5%). Early-stage applications produced stronger benefits overall. Principal component analysis distinguished treatment effects, with UI+B samples clustering consistently apart from controls. These results demonstrate that combining biostimulants with urease inhibitors can simultaneously improve yield, quality, and sustainability in malting barley, supporting reduced nitrogen input in Mediterranean systems.

Keywords: Malting barley; Biostimulants; *Ascophyllum nodosum* extract; Nitrogen fertilization; Grain Plumpness; Grain protein concentration; Mediterranean Conditions

1. Introduction

Barley (*Hordeum vulgare* L.) stands as one of the most important cereal crops globally, serving both food and feed sectors and occupying a particularly crucial role in the malting and brewing industries [1,2]. The economic value of barley for malting purposes depend on key quality attributes, notably mean grain size, grain size distribution, and grain protein concentration [3,4]. Among these, grain plumpness holds particular significance, with larger kernels positively correlated with malt extract yield—directly impacting beer production volume and profitability [3–6]. However, achieving consistently high kernel size remains challenging, particularly under Mediterranean and other stress-prone environments where thin grains are often produced.

Grain protein concentration (GPC) is another decisive quality parameter. Optimal GPC, typically ranging between 9% and 12%, is required to ensure proper enzymatic activity during malting, efficient yeast nutrition, and high extract yield [7–9]. Both deficient and excessive protein levels compromise malting quality, emphasizing the necessity of precise agronomic and genetic control. Moreover, grain size is a critical quality criterion, with acceptable standards typically requiring more than 90% of kernels to be retained above a 2.2 mm sieve [10]. Grain plumpness ensures higher malt extract yield and is closely linked to overall brewing performance [3,11].

The yield and quality performance of barley is significantly affected by the interactions among genotype, environment, and treatment [5,12,13]. Phenotypic plasticity, defined as the environmentally responsive generation of various phenotypes from a single genotype [14], is crucial for the adaptation of barley to a range of environmental conditions. Genotypes exhibiting high stability are valuable for securing predictable yields under stress-prone environments, while those with pronounced plasticity may exploit favorable conditions to achieve superior performance [5,15].

Nevertheless, efforts to simultaneously improve grain yield and quality face significant biological constraints. Compensatory relationships between yield components, such as the negative association between grain size and protein concentration, often impede progress [5,16]. Abiotic stresses, including drought, extreme temperatures, and nutrient deficiencies, further exacerbate these challenges, particularly under rainfed and marginal agricultural systems [17,18].

Among agronomic factors, nitrogen (N) management is particularly influential. Nitrogen is crucial for the growth of plants; however, the nitrogen use efficiency (NUE) in cereal crops remains relatively low, with recovery rates of applied nitrogen varying between 26% and 68% [19]. Low NUE not only limits yield potential but also leads to environmental degradation through nitrate leaching, ammonia volatilization, and nitrous oxide (N_2O) emissions [20,21]. It is essential to implement management strategies to mitigate N_2O emissions in agriculture [21], a potent greenhouse gas with a global warming potential 265 times that of carbon dioxide [19].

Effective nitrogen management aims to optimize yield and quality while minimizing environmental impacts. Excessive application of N increases GPC beyond acceptable limits for malting, thereby reducing extract yields and product quality [7]. On the other hand, insufficient application may compromise yield and soil health, particularly in intensive cropping systems [19]. Moreover, climatic variability, particularly rainfall patterns following fertilizer application, exerts a strong influence on nitrogen dynamics, uptake efficiency, and final grain attributes [9,22].

The "4R" nutrient stewardship framework—applying the right fertilizer source at the right rate, time, and place—has been advocated to enhance NUE and environmental sustainability [19,23]. However, the practical application remains challenging, particularly due to the growing unpredictability of weather patterns influenced by climate change.

Urea, representing about 80% of the straight nitrogen fertilizer market, remains the most widely applied nitrogen form due to its high nitrogen content and affordability [24,25]. However, urea is prone to rapid hydrolysis into ammonium, resulting in substantial ammonia (NH_3) volatilization losses. The application of urease inhibitors (UIs) presents an effective strategy to slow hydrolysis, enhancing nitrogen retention and reducing emissions [24–26]. Literature suggest that UIs can decrease NH_3 losses by up to 94%, although their impact on grain yield remains modest and context-dependent [25,27–29].

Subsequent progress involves breeding techniques and genetic engineering approaches designed to enhance intrinsic NUE characteristics. Selective breeding has focused on traits such as greater root length or surface area, increased above-ground biomass, higher harvest index, and enhanced photosynthetic capacity [30–35]. Genetic modification strategies have also been employed to manipulate genes associated with nitrogen uptake, assimilation and remobilization [36–40]. Nonetheless, the majority of these genetically modified plants have primarily been tested in controlled or hydroponic settings, leaving their efficacy in diverse field conditions unverified [38,41,42].

An additional avenue for improving crop performance under reduced nitrogen inputs involves the application of biostimulants, which in most cases are plant-derived products. Biostimulants are defined as biological substances or microorganisms applied to plants to enhance nutrient efficiency, stress tolerance, and/or crop quality traits [43–45]. Seaweed extracts, particularly from *Ascophyllum nodosum*, have emerged as leading products in the biostimulant market due to their wide range of beneficial effects on plant physiology [46–50].

Seaweed extracts contain complex mixtures of bioactive compounds, including phytohormones (cytokinins, auxins, abscisic acid), polysaccharides, amino acids, vitamins, and phenolic compounds [47,51,52]. These compounds are reported to improve plant vigor, promote root development, enhance photosynthetic activity, delay senescence, and increase tolerance to abiotic stresses such as drought, salinity, and temperature extremes [50,53–55]. In cereals such as barley, the application of seaweed extracts has been associated with increases in grain yield and reductions in grain nitrogen content, suggesting a potential dual benefit for both yield and malting quality optimization [7].

Despite the promising results, the adoption of seaweed-based biostimulants in cereal production faces challenges. Variability in product composition, inconsistency in crop responses, and limited large-scale field data hinder broader commercial uptake (48,50). Standardization of biostimulant formulations and rigorous agronomic validation under diverse field conditions are necessary to fully realize their potential benefits.

Building on the promising outcomes observed with the use of urease inhibitors and biostimulants in enhancing NUE, we hypothesize that further optimization of barley production sustainability can be achieved by integrating biostimulant applications with advanced nitrogen fertilization strategies. This hypothesis is grounded in evidence suggesting that biostimulants, particularly seaweed-based extracts, not only improve plant nutrient uptake and stress resilience but may also modulate grain protein concentration and increase grain yield under reduced nitrogen regimes [7,48,50]. Furthermore, combining biostimulants with enhanced efficiency fertilizers, such as those incorporating urease inhibitors, could synergistically reduce nitrogen losses while maintaining or even improving crop performance [24,25]. Therefore, the aim of this study was to evaluate the combined effects of applying a biostimulant (seaweed extract) and different nitrogen fertilization approaches (i.e., common urea, urea with a urease inhibitor) at different distinct growth stages of malting barley cultivation, under varying climatic conditions, focusing on both quantitative and qualitative characteristics.

2. Materials and Methods

2.1. Site Description

The experiments were carried out for two growing seasons (2022- 2023 and 2023-2024) in two areas of Thessaly with different climatic conditions, namely Farsala and Almyros, in commercial fields. Due to the different climatic conditions encountered in the two areas, the area of Farsala will be referred to as Environment 1 (E1) (39°15'44"N 22°28'34"E) and Environment 2 (E2) (39°14'57"N 22°29'34"E) for the growing seasons 2022- 2023 and 2023-2024, respectively. Similarly, for the Almyros area, Environment 3 (E3) (39°09'22"N 22°47'02"E) and Environment 4 (E4) (39°12'22"N 22°42'49"E) for the 2022- 2023 and 2023-2024 growing seasons, respectively. Sowing took place on 19 November, 20 November, 1 December and 2 December for E1, E2, E3 and E4, respectively. Seeding rate was 180 kg per hectare, with row spacing of 12 cm. Two malting barley genotypes were evaluated: G20 as early-maturing and G45 (Ackermann Saatzucht GmbH & Co. KG, Irlbach, Germany) as late-maturing. The previous cultivations were malting barley for E1 and E3, vetch and watermelon for E2 and E4, respectively. Soil properties are presented on Table 1. A total of 40 kg N/ha and 40 kg P_2O_5/ha were applied in basic fertilization (20-20-0 +22SO₃), in all environments. Finally, harvest took place on 10 June 2023, 2 June 2024, 25 May 2023 and 25 May 2024 for E1, E2, E3 and E4, respectively, at seed moisture of 9% to 11.5%.

Table 1. Soil physicochemical properties at the four experimental environments (E1–E4). Values represent measurements of organic matter content, pH, total nitrogen, available phosphorus, and exchangeable potassium.

	Soil Properties					
	Soil Texture	Organic matter (%)	pH	Total Nitrogen (%)	Phosphorus (ppm)	Potassium (ppm)
E1	Clay	2.02	7.05	0.147	20.12	266
E2	Clay	2.34	7.10	0.161	23.66	224
E3	Clay loam	1.95	7.20	0.133	19.57	172
E4	Clay loam	1.86	7.22	0.129	16.53	281

2.2. Treatments and Experimental Design

The experiments were arranged in a randomized complete block design (RCB), with three replicates in each treatment. The plot size for each treatment was 40 m² in E1 and E2, and 39 meters in E3 and E4. The treatments applied as surface fertilization, were as follow: i) common sulfur urea (U), ii) common sulfur urea with addition of biostimulant (U + B), iii) urea with urease inhibitor (UI), iv) urea with urease inhibitor with addition of biostimulant (UI + B) and v) without surface fertilization as control. For the treatments using common sulfur urea 90kg N/ha was applied (40-0-0 +14SO₃). For the treatments with urea with urease inhibitor, 7.5 kg N/ha were (40-0-0 + 5.7S) The biostimulant that was used came from *Ascophyllum nodosum* Extracts (ANE) from marine algae Goëmar GA142 (0-31-5). The dosage applied was 1L/ha with a 1:50 dilution.

The application of the biostimulant and surface fertilizer was conducted at the end of tillering (Z24) and at the beginning of stem elongation (Z30), respectively G45, and these will be considered as early stages of application. For G20, applications of biostimulant were performed at the beginning of stem elongation (Z30) and surface fertilization at mid-stem elongation (Z32), and these applications are considered to have been applied at mid-stages.

2.3. Measurements

2.3.1. Growth Rate

To determine the Growth Rate, phenology of two genotypes was monitored every 10 days for the beginning of tillering to the stage of anthesis and every 5 days from anthesis to harvesting, utilizing the scale of Zadoks et al., [56], following the average phenology in each plot.

2.3.2. Yield and Yield Components

To determine yield and its components, 6 quadrats of 0.25 m2 were manually sampled from its plot following a "Z" pattern. The data regarding aboveground biomass, Yield and Harvest Index (HI) were derived from these samples, subsequently converted to tn/ha. Spikes/m2 were determined from the samples in each plot. To determine the number of grains per spike, ten spikes were randomly chosen from each sample. The thousand grains weight (TGW) was determined from three samples per plot, consisting of 100 clean grains, which were counted, and their weigh was raised to 1000 grains. For the determination of yields and TGW the humidity of the grains was deduced to 11% in all samples.

2.3.3. Grain Size

Analytica EBC "Sieving test for Barley" method was used for the determination of grain size fraction using a screening machine equipped with three slotted sieves with different diameter (2.8mm, 2.5mm and 2.2mm). Three samples of 100g of grains per plot were placed on the top sieve (2.8mm) and shaken for 5 minutes. Then the grants that remained in each sieve were weighted in a presession scale (0.01g). Each sample followed a classification into three grain fractions: >2.8mm, >2.5mm (retention fraction) and >2.2mm (maltable fraction).

2.3.4. Grain Protein Content

Grain Protein Content (GPC) was determined, from 3 samples per plot and from measuring the nitrogen content using the Kjeldahl method and multiplying the results with factor 6.25.

2.4. Statistical Analysis

Prior to conducting ANOVA, standardised residuals were examined with the Shapiro-Wilk test to check whether the data follows normal distribution. This was followed by a general ANOVA on pooled data to determine the effects of Environments, Genotypes, Treatments and their interactions on the yield and qualitative traits, using Tukey's multiple range test. After Environment was identified as having a statistically significant effect on the traits examined, further ANOVA was conducted on the effect of the interaction in each environment. All statistical analyses were performed using STATA now 18 MP (StataCorp LLC, 2023). In addition, Principal Component Analysis (PCA) was performed to explore the multivariate structure of treatment responses across different environments and application stages in malting barley. The analysis included 10 agronomic and quality-related traits: aboveground biomass, grain yield, spikelets per square meter, thousand grain weight (TGW), harvest index (HI), grains per spike, grain protein content (GPC), maltable fraction, retention fraction, and grain size fraction >2.8 mm. Two separate Principal Component Analysis (PCA) were conducted corresponding to the two application stages of treatment: early stages and mid-stages. Each analysis included 80 observations, derived from factorial combinations of treatments, genotypes, and environments. Data visualization and modeling for PCA were performed using JMP Pro, version 18 (SAS Institute, 2023). Significant differences between treatment means were compared using the protected least significant difference (LSD) procedure at P<0,05.

3. Results

3.1. Meteorological Data

Regarding the climatic conditions, significant differences were observed between the regions and growing seasons. In the 2022-2023 growing season, E1 exhibited ideal conditions for malting barley cultivation, while at E3, the conditions were considered unfavorable for the Thessaly region (Figure 1A). E3, the average temperature was higher by 2.13°C to 5.32°C throughout the growing season compared to E1 (Figure 1A,C). Maximum temperature (T_{max}) did not show significant differences between the two regions, with temperatures ranging from 9.21°C to 26.78°C at Environment 1 and from 10.2°C to 25.5°C at E3 (Figure 1A,C). In the second growing season, E4 presented more favorable conditions for the cultivation of barley. Regarding average air temperature (T_{mean}), no differences were found between the two regions, with temperatures ranging from 3.68°C to 24.3°C at E2 and from 4.13°C to 20.1°C at E4 (Figure 1D). The recorded total precipitation was 291,4mm, 258,7mm, 276mm and 334,4mm at E1,E2,E3 and E4, respectively (Figure 1A–D).
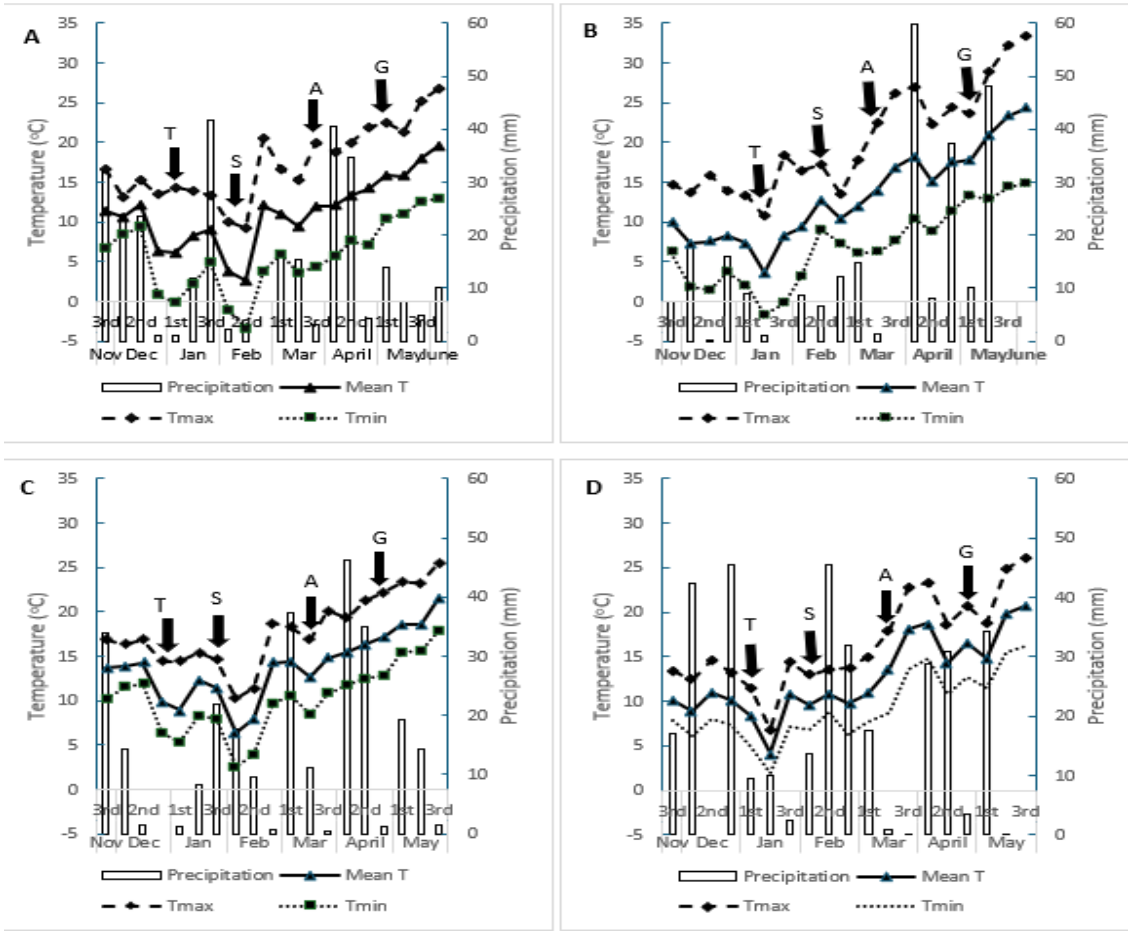
Figure 1. Diagrams with the meteorological data by enviroment with A,B,C and D, Enviroment 1, Enviroment 2, Enviroment 3 and Enviroment 4, respectively. Temperature (oC), maximum, minimum and average and precipitation (mm) of the genotypes per decade during the biological cycle are shown. Arrows denote key phenological stages: T= Tillering; S= Stem elongation; A= Anthesis; G= Grain filling.

3.2. Impact of Treatments on Quantitative Traits

The statistical analysis revealed a significant effect of the environment on aboveground biomass, yield, harvest index (HI), number of spikelets per square meter, and the weight of thousand grains (TGW), but no significant effect on grains per spike (Table 2). Both genotypes and treatments had a significant impact on all quantitative traits (p-value < 0.01). Furthermore, interactions between the factors Environment*Genotype (G*E) significantly influenced all yield components (Table 2). The interactions of the factors Environment*Treatment (E*T) and Genotype*Treatment (G*T) did not affect aboveground biomass, HI, spikelets per square meter, and TGW (Table 2). The interaction of all three factors (E*G*T) played a significant role in aboveground biomass, yield, spikelets per square meter, and grains per spike.

Among the environments, E1 exhibited higher values for aboveground biomass, yield, spikelets per square meter, and TGW in G20 and G45. The lowest values for quantitative traits were observed in E4 for G45, and in E2 for G20 (Table 3). ANOVA conducted across environments revealed differences in the effects of genotypes and treatments on quantitative traits, with statistically significant differences in all cases (Tables 3 and 4). HI in E1 was the trait that did not show significant differences in the G*T interaction (Table 3).

Table 2. Interaction of enviroment, genotypes and treatments on quantitative traits (Aboveground Biomass, Yield, Harvest Index (HI), Spikelets per square meter and Grains*Spike^{-1}).

	Quantitative Traits					
	Aboveground Biomass	**Yield**	**HI**	**Spikelets*m^{-2}**	**TGW**	**Grains* Spike^{-1}**
Environment (E)	***	***	***	***	***	ns
Genotype (G)	***	***	***	***	***	***
Treatment (T)	***	***	*	***	***	**
E*G	***	***	***	***	***	**
E*T	***	***	***	ns	ns	***
G*T	ns	**	ns	**	**	***
E*G*T	**	***	ns	*	**	***

***=p<0.001,**=p<0.01,*=p<0.05 and ns: no significant.

3.2.1. Effects of Biostimulant and Fertilization at Early Stages on Quantitative Traits

The biostimulant ANE and surface fertilization treatments were applied at the mid-tillering stage (Z24) and at the beginning of stem elongation (Z30), respectively, on G45. The UI+B treatment was the most effective across all environments. Specifically, in favorable conditions (E1), G45 showed increases compared to the control in aboveground biomass (53.88%), yield (64.94%), spikelets per square meter (47.4%), TGW (22.37%), and grains per spike (15.22%) (Tables 3 and 4). In E4, which presented the lowest overall values, UI+B led to increases in G45 of 76.82% for aboveground biomass, 78.51% for yield, 50.37% for spikelets per square meter, 16.89% for TGW, and 9.61% for grains per spike.

Among the treatments U, U+B, and UI, no significant differences were observed for most quantitative traits across all environments. However, TGW exhibited statistically significant differences between UI and U in Environments 1 and 2 for G45. Additionally, a significant difference in yield between U and UI was observed for G45 in E4. Despite the limited statistical significance in many cases, there was a clear tendency for increases under the UI treatment, particularly in yield (up to 53.05%) and TGW (16.94%) in E1, and spikelets per square meter (up to 42.54%) in E4 (Tables 3 and 4).

3.2.2. Effects of Biostimulant and Fertilization at Mid-Stages on Quantitative Traits

The biostimulant ANE was applied at the beginning of stem elongation (Z30-31), and surface fertilization at mid-stem elongation (Z32-33), on G20. In most cases, no significant differences were observed between treatments, with the exception of the control group. However, the UI+B and U+B treatments demonstrated the highest effectiveness for G20 across all environments.

Statistically significant differences were observed only for grains per spike in E1 and TGW in Environment 2, with UI+B showing the highest values for G20. Additionally, aboveground biomass and spikelets per square meter were consistently enhanced by the U+B treatment across all environments. The highest percentage increases for G20 were recorded in Environment 2, with aboveground biomass increasing by 89.82% and spikelets per square meter by 62.28% compared to the control. Under favorable conditions in E1, aboveground biomass and spikelets per square meter increased by 53.23% and 27.77%, respectively.

In contrast, yield, TGW, and grains per spike were most effectively enhanced by the UI+B treatment for G20. Specifically, in E1, UI+B led to increases of up to 45.06% in yield, 17.26% in TGW, and 12.24% in grains per spike compared to the control. In Environment 2, the same treatment significantly improved yield (92.22%), harvest index (HI) (17.78%), TGW (16.57%), and grains per spike (10.37%) for G20. In Environments 3 and 4, the UI+B treatment remained the most effective for G20. However, in E4, the control treatment exhibited the highest HI value for G20.

Table 3. Interaction between genotypes and treatments at Environments 1, 2, 3, 4 on aboveground biomass, yield, Harvest Index (HI), according to Fisher test (p-value<0.05). Analysis of variance (ANOVA) is also shown.

		Aboveground Biomass				Yield				HI			
		E1	E2	E3	E4	E1	E2	E3	E4	E1	E2	E3	E4
G45	U	12.85ab	6.46ad	11.25abc	6.72bc	6.68cd	3.19ac	6.13b	3.28ac	0.52	0.43	0.55cde	0.49
	U+B	15c	8.24ab	11.9ab	8abc	6.74cd	3.5ab	6.99c	3.49ac	0.45	0.43	0.58ce	0.44
	UI	12.25b	8.45abc	11.3abc	8.62ab	7.18c	3.68ab	7.07c	3.52ac	0.58	0.47	0.63c	0.40
	UI+B	13.85abc	9.45bc	12.05ab	9.46ad	7.62c	4.02abd	7.36c	4.32ab	0.55	0.51	0.62c	0.46
	Control	9d	5.51d	9.65c	5.35c	4.62ae	2.51c	4.91d	2.42c	0.51	0.44	0.51ade	0.49
G20	U	13 ab	7.25abd	12.4ab	12.16dei	5.26ab	3.44abc	5.43ad	4.63b	0.41	0.47	0.44ab	0.38
	U+B	14.25ac	10.63c	12.65a	14.17e	5.43ab	4.24bd	5.73ab	4.65b	0.38	0.39	0.45ab	0.32
	UI	13.1abc	8.46abc	12.15ab	10.08ad	5.33ab	3.35abc	5.68ab	3.65a	0.41	0.39	0.44ab	0.36
	UI+B	14.2ac	9.24bc	12.35ab	13.54e	5.73bd	4.94d	5.88ab	4.69b	0.40	0.53	0.49ad	0.30
	Control	9.3d	5.6d	10.45bc	9.03ab	3.95e	2.57c	3.86e	3.49ac	0.42	0.45	0.37b	0.39
ANOVA													
Genotype (G)		*	***	**	***	***	*	***	**	***	ns	***	*
Treatment (T)		***	***	***	***	***	***	***	***	ns	*	***	ns
G*T		*	*	*	***	*	**	*	***	ns	ns	*	ns

***=p<0.001,**=p<0.01,*=p<0.05 and ns: no significant.

Table 4. Interaction between genotypes and treatments at Environment 1 (E1), Environment 2 (E2), Environment 3 (E3) and Environment 4 (E4) on Spikelets*m^{-2}, thousand grains weight (TGW), grains*spike^{-1}, according to Fisher test (p-value<0.05). Analysis of variance (ANOVA) is also shown.

		Spikelets*m^{-2}				TGW				Grains*Spike^{-1}			
		E1	E2	E3	E4	E1	E2	E3	E4	E1	E2	E3	E4
G45	U	745c	423abc	657b	448bc	38.80c	35.60d	38.59ade	35.4ac	29.5ab	27.8a	27.5ae	28.3abc
	U+B	822cd	476abd	736bc	546abc	42.48bd	39.45a	39.97abd	35.7ac	28a	27a	28a	28.8ab
	UI	834cd	478abd	691bc	583ab	44.17ab	40.52a	40.22abd	36.92a	29.3ab	27.7a	28.3ac	28.8ab
	UI+B	880d	535d	768c	615a	46.22a	42.40ab	41.64abc	38.26ad	28a	28ac	31bc	28.5ab
	Control	597a	371ace	543a	409c	37.77c	34.65d	35.64e	32.73c	24.3c	26.2a	25de	26c
G20	U	582ab	368ce	531a	552ab	44.21ab	43.92bc	40.99abc	40.75bd	30.2ab	28.2ac	30.3abc	28abc
	U+B	623a	456abcd	567a	584ab	44.66ab	44.40bc	43.11bc	42.85b	27.5ad	30.5b	31.2b	30bd
	UI	576ab	410abc	522a	480ab	44.71ab	44.49bc	41.20abc	40.95bd	27.8a	29.8b	29abc	29.2
	UI+B	612a	486bd	546a	551abc	47.13a	46.92c	43.86c	43.67b	31.5b	31b	31.3b	32d
	Control	479b	281e	500a	467bc	40.49cd	40.25a	37.52de	37.3a	24.5cd	27a	22.8d	27.3ac
	ANOVA												
	Genotype (G)	***	**	***	*	***	***	**	***	ns	***	*	***
	Treatment (T)	***	***	***	**	***	***	***	***	***	***	***	***
	G*T$_e$	*	*	**	*	**	*	ns	**	*	**	**	**

***=p<0.001,**=p<0.01,*=p<0.05 and ns: no significant.

3.3. Impact of Treatments on Qualitative Traits

Ensuring the quality characteristics of malting barley cultivation is a primary objective for the brewing industry. Aiming to more precise investigation of the optimal fertilization scheme on the key qualitative traits (GPC and maltable fraction). Aiming to more precise investigation of the optimal fertilization scheme on the key qualitative traits (GPC and maltable fraction), there was evaluated beyond the desired criterion for malting (> 2.2 mm), two advance criteria in terms the grain size. Specifically, grain sizes higher than 2.5 mm and 2.8 mm were considered. Statistical analysis revealed that all three factors (environment, genotype, and treatment) had a significant effect on all quality traits examined (p-value < 0.0001) (Table 5). Significant two-factor interactions (E*G, E*T, and G*T) were observed. Furthermore, three-factor interactions, as well as the interaction involving all factors, also showed significant effects on all quality traits (p-value < 0.0001) (Table 5).

An ANOVA was performed to evaluate the impact of genotypes and treatments on the qualitative traits in each environment. Both factors had a noticeable influence on GPC and grain size, with p-values < 0.0001 for both genotypes and treatments, except for genotype effects on maltable in E3 (p-values < 0.01). Statistically significant differences were observed for the interaction between genotypes and treatments (G*T), affecting all qualitative traits, except for maltable fraction in E3 (Table 6). Maltable fraction, retention fraction, and grain sizes larger than 2.8 mm were favored in Environments 1 and 3, while the lowest values were observed in E4 (Table 6). For GPC, E1 exhibited the highest values within the acceptable limits. In Environments 2, 3, and 4, there was no consistency observed for this characteristic. However, the UI+B treatment appeared to push GPC to the desired levels (Table 6).

Table 5. Interaction of enviroment, genotypes and treatments on qualitative traits (grain protein content (GPC), and sieving test).

	Qualitative Traits			
	GPC	Maltable (>2.2mm)	Retention (>2.5mm)	>2.8mm
Environment (E)	***	***	***	***
Genotype (G)	***	***	***	***
Treatment (T)	***	***	***	***
E*G	***	***	***	***
E*T	***	***	***	***
G*T	***	***	***	***
E*G*T	***	***	***	***

***=p<0.001,**=p<0.01,*=p<0.05 and ns: no significant.

Table 6. Interaction between genotypes and treatments at Environments 1, 2, 3, 4 on grain protein content (GPC) and sieving test, according to Fisher test (p-value<0.05). Analysis of variance (ANOVA) is also shown.

		GPC (%)				Maltable (>2.2mm) (%)				Retention (>2.5mm) (%)				>2.8mm (%)			
		E1	E2	E3	E4	E1	E2	E3	E4	E1	E2	E3	E4	E1	E2	E3	E4
G45	U	7.96a	9.78c	9.46ab	11.06abc	98.31ab	92.05a	98.89ab	75.45b	87.61c	49.82c	88.62d	22.46de	56.82c	6.46a	54.63cde	1.91a
	U+B	8.61bc	10.34cd	11.22de	9.09de	98.93a	92.28a	98.99ab	76.53bc	89.45a	53.29c	89.31cd	29.29de	60.06b	8.66abc	57.37cd	3.80ac
	UI	9.77a	11.08ae	9.28a	11.99cf	98.97a	92.35a	98.98ab	81.88cd	89.79a	67.17a	90.55cd	37.99d	61.39b	11.28bc	59.15bcd	0.93a
	UI+B	10.33d	10.47be	10.71cd	10.25ae	99.06a	93.58ab	99.06b	86.57d	90.67a	70.03a	98.74a	38.91d	64.42ab	12.61c	62.11bc	0.95a
	Control	6.95c	7.44e	7.66b	7.95d	96.31b	88.85f	98.43cd	74.68b	84.54b	40.62d	84.04e	11.66f	42.18d	5.57a	41.71	0.71a
G20	U	9.95ac	11.71ab	13.55e	10.69ab	98.59a	95.37bcd	98.69ad	94.41a	86.83c	84.33e	92.48c	74.45ab	60.74b	28.73d	67.1abc	24.40a
	U+B	9.88a	11.95b	10.70d	11.85bcf	98.99a	96.72cde	98.7ab	95.79a	89.51a	86.43be	92.95bc	77.15a	65.07ab	31.84d	69.09ab	26.11a
	UI	9.80a	12.78d	9.89c	12.73f	99.03a	97.21de	98.82ab	96.02a	89.79a	89.65b	93.24bc	77.78a	66.11a	50.74e	70.4ab	17.35b
	UI+B	10.95d	11.39ab	11.31de	10.93abc	99.09a	97.61de	98.89ab	95.62a	90.47a	90.59b	93.71bc	79.87a	67.40a	56.56f	72.45a	27.44a
	Control	8.18bc	9.08c	9.40ab	9.14de	96.48b	94.86bc	98.12c	86.01d	84.41b	66.33ad	88.21d	68.35c	48.09d	7.49ab	52.66e	15.12b

ANOVA

Genotype (G)	***	***	***	***	***	***	**	***	**	***	***	***	***	***	***	***
Treatment (T)	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
G*T$_e$	***	***	***	***	**	***	*	**	**	***	***	***	*	***	**	***

Effects of Biostimulant and Fertilization at Mid-Stages on Qualitative Traits

The UI+B and U+B treatments had a more pronounced effect on the qualitative traits of the very early genotype G20, as the biostimulant ANE was applied at the beginning of stem elongation (Z30–31) and surface fertilization at mid-stem elongation (Z32–33). A differential response was observed between treatments, with G20 responding more favorably to UI+B. No statistically significant differences were observed between U, U+B, UI, and UI+B treatments for maltable grain content in G20, as all treatments met the industry-accepted limits across all environments. Under favorable conditions (E1), the UI+B treatment led to a 19.31% increase in grain size >2.8 mm and a 6.06% increase in retention fraction compared to the control (Table 6). The highest values for both traits were observed in E3. Although the lowest values for all grain size categories were recorded in E4, UI+B treatment still increased these values in G20 by 9.61% for maltable grain, 9.43% for retention fraction, and 9.28% for grain size >2.8 mm, relative to the control. Regarding grain protein content (GPC), G20 showed relative stability across all treatments (U, U+B, UI, UI+B), but only the UI+B treatment consistently met the acceptable GPC limits across all environments (Table 6).

Among the other treatments, U+B and UI had similar effects on grain quality traits in G20. Statistically significant differences were observed for grain size greater than 2.8 mm in E2 and E4, and for GPC in E2 and E3 (Table 6).
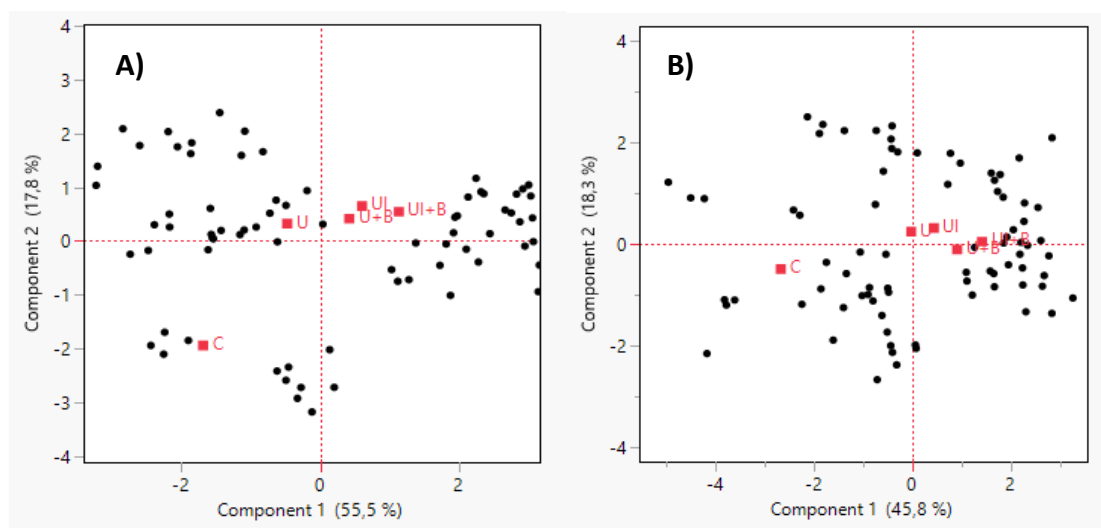
3.4. Principal Component Analysis

Principal Component Analysis (PCA) was conducted to assess the multivariate response of treatment effects on quantitative and qualitative traits when applied at early and mid-stages. The analysis was based on 10 quantitative traits, including:
aboveground biomass, grain yield, spikelets per square meter, thousand grain weight (TGW), harvest index (HI), grains per spike, grain protein content (GPC), maltable fraction, retention fraction, and grain size fractions >2.8 mm. total of 80 observations were included in each analysis.

At the first application stage (Z30–31), the first two principal components explained 73.3% of the total variance (PC1: 55.5%, PC2: 17.8%) (Figure 2A). In the second stage (Z32–33), the total variance explained was 64.1% (PC1: 45.8%, PC2: 18.3%) (Figure 2B). In both PCAs, the control treatment (C) was clearly separated along PC1 from the other treatments, suggesting a consistently distinct performance pattern across multiple traits. Treatments including surface fertilization and/or biostimulants (U, UI, U+B, UI+B) clustered on the positive side of PC1, indicating a shared and favorable effect on early plant development.

At early stages, treatments formed a more compact cluster, indicating a uniform multivariate response to early application. In contrast, the PCA at mid-stages showed greater dispersion among treatments, particularly along PC2, suggesting the emergence of additional variation, possibly due to environment-dependent responses or genotype-by-treatment interactions (Figure 2B).

Overall, the PCA results suggest that biostimulant and fertilization treatments, particularly UI+B, consistently enhanced early plant performance regardless of application timing. However, application at earlier stages appeared to produce a more cohesive and pronounced multivariate response, underscoring the importance of timing in optimizing early crop vigor.

Figure 2. A,B. Principal component analysis (PCA) conducted for all traits, quantitative and qualitative, based on A) early and B) mid-stages of biostimulant and surface fertilization application. Total number of samples was 80, in 4 Enviroments for the genotype G45. Red dots corresponds to a treatment   and the black dots   the 10 variables were yield, aboveground biomass, harvest index (HI), thousand grains weight (TGW), grain protein content (GPC), maltable, retention fraction and grain size greater than 2.8mm. U= Urea, U+B= Urea and biostimulant *Ascophyllum nodosom* extract, UI= Urea with inhibitor urease, UI+B=   Urea with inhibitor urease and biostimulant *Ascophyllum nodosom* extract and C= Control without surface fertilization.

The results of the present study provide a detailed investigation of the impact of climatic conditions, treatment's stage (represented by the different genotypes), and fertilization treatments, on both yield and quality traits of malting barley. Five treatments were applied: simple urea (U), U with biostimulant *Ascophyllum nodosum* extract (ANE) (U+B), urea with urease inhibitor (UI), UI combined with biostimulant ANE (UI+B), and control, without surface fertilization and ANE, across four environments, on two genotypes (G20 and G45). The findings underscore the complex interplay between environmental factors, genotypic characteristics, and agronomic practices in determining barley productivity and quality.

3.4.1. Impact of Environmental and Treatment Factors at Early Stages

Grain yield, in combination with key yield components such as aboveground biomass, harvest index (HI), spikelets m², and grains per spike, were used to measure and interpret the results [57]. In malting barley, aside from achieving high yields, specific quality criteria must be met regarding grain protein content (GPC) and grain size to satisfy industrial requirements. In terms of quality traits, the results of this study emphasize the importance of selecting the right genotype and fertilization strategy to meet the specific needs of the malting industry. Grain protein content (GPC) in malting barley is a critical quality trait, with an optimal range between 9.5% and 11.5%, while the retention fraction (proportion of grains larger than 2.5 mm) must exceed 90% [9].

In E1, more favorable climatic conditions were observed for G45, which exhibited the highest values for both quantitative and qualitative traits. The amount of rainfall (291.4 mm) and its distribution, combined with milder temperatures (Tmean = 11.17°C) during the growing season, and the low temperatures during the grain filling stage, made E1 the most ideal for malting barley cultivation, findings that align with existing literature [58,59].

Less favorable conditions prevailed in E4, where G45 had the lowest values for productive and quality traits. Although temperatures during the critical stages of growth were mild and rainfall (334.4 mm) was adequate, it is likely that the genotypes suffered from lodging after the rainfall (33.9 mm) that occurred in the first ten days of May (Figure 1D). The high-water availability during the vegetative stages of the crop led to an increase of the plants' height due to the rate of cell division

[60]. In contrast, in Environment 2, the lower rainfall during the vegetative stages, resulted shorter plants more resistant to lodging, especially during   the greater rainfall in May in this environment. However, in E2 reduction in grain size was observed which may be attributed to drought stress during late grain filling, factor which restricts carbohydrate incorporation in the grain, leading to a decrease in grain size despite consistent total nitrogen content [19]. The sensitivity of barley cultivars to both temperature extremes and the timing of precipitation with impact on yield components and quality characteristics have also been stated from other researchers [61–63].

The application of the biostimulant ANE and surface fertilization at early stages, had a pronounced effect on both quantitative and qualitative traits, particularly on G45. Among the different treatments, UI+B showed the most consistent and significant improvements on traits like aboveground biomass, yield, and thousand-grain weight (TGW) across all environments. This occurred, even though the UI+B treated at the lower dose (15 kg N/ha), compared   the simple urea treatments (U and U+B). The combination of urea with urease inhibitors (NBPT) and the biostimulant ANE has been extensively studied, with positive effects observed in crops [64–67]. However, little is known about how ANEs influence nitrogen (N) uptake and assimilation mechanisms under reduced N conditions [65,68]. The integration of biostimulants with current farming practices to enhance NUE may present challenges (e.g., method of application, timing, rates) in some production systems [65,69,70]. Nevertheless, the addition of ANE played a significant role in positively influencing plant growth, particularly by stimulating root development and nutrient uptake, likely facilitating the observed improvements in yield. Specifically, field experiments showed that foliar application of engineered ANE biostimulants maintained or increased grain yield in barley grown with 25% less N fertilizer [65]. Maignan et al., [71], report that ANE significantly improved grain yield (+3.59 tn/ha), total grain N, and NUE. ANE application improved growth during tillering and stem elongation increased the number of fertile tillers, grain number per m², and TGW [69,71,72].

The positive effects of UI+B sign that this treatment can lead to reducing excessive N fertilizer application, by increasing the nutrient use efficiency (NUE), with ecological and economic benefits for the environment and the   farmers respectively [73,74]. Similarly, Maignan et al., [71] and Abalos et al. [26], report that NBPT had a significantly positive effect on grain yield in barley and wheat, respectively. This may be due to the alkaline pH of the soil in all four areas, where the urease inhibitor NBPT had the most significant effect (Table 1) option which in in accordance with Abalos et al., [26]. Other studies show that the urease inhibitor (NBPT) in temperate grasslands reduce NH_3 losses from surface-applied urea [18,75,76] and increase yield and N uptake [75,77]. Adding urease inhibitor NBPT to urea significantly increased yields and N uptake compared to standard urea, confirming the findings of previous studies [75,76,78,79].

Regarding qualitative traits such as grain protein content (GPC) and grain size, the application of treatments played a significant role in meeting malting industry standards. Specifically, the UI+B treatment resulted in notable improvements in grain size (greater than 2.8 mm) and retention fraction, which are critical for the malting process. In particular, E1 demonstrated the highest quality traits, with UI+B achieving significant increases in grain size and retention fraction. Similarly, in the less favorable E4, where lower values were observed for these traits, the combination of urease inhibitor and biostimulant (UI+B) treatment helped stabilize and elevate GPC to the desired levels. These findings demonstrate that the UI+B treatment has the potential to improve quality traits, even under less-than-ideal growing conditions. Additionally, the biostimulant, regardless of fertilizer type, led to increased grain size, with UI+B showing the most noteworthy improvements across all treatments. This suggests that the combination of urease inhibitor and biostimulant not only improves efficiency traits but also enhances malting quality by increasing grain plumpness. Furthermore, GPC remained stable across most treatments, with UI+B proving to be the most effective in maintaining GPC within the desired limits. This is particularly important for the brewing industry, as maintaining high-quality malting barley is essential for optimal beer production [80,81].

The aforementioned results are likely a consequence of ANE's ability to generally stimulate root growth and increase the efficiency of soil nutrient absorption and utilization [82,83]. An increase in

nutrient uptake efficiency was also observed in cereals (H. vulgare, Triticum aestivum/durum, Zea mays). In malting barley, a larger root system increases starch content and malt extract during the dry season [82,84]. In contrast, a weak root system under dry conditions leads to low grain yield and a significant deterioration in quality [82,84]. Moreover, Goettl et al. [85] report that N plays a role in grain size. However, the literature supports the notion that GPC is inversely associated with grain size [16,19]. This is because starch accumulation appears to be more sensitive to post-anthesis stress than nitrogen accumulation [19,86]. In this study, this claim was not confirmed, as climate conditions and genotype played a more important role [87–89]. It is worth noting that the addition of ANE improved plant tolerance in environments where stressful growing conditions prevailed, something which have been stated by other researchers [66,82,83,90]. The effects of biostimulants on plant defense and adaptation to abiotic stresses include, initially, the absorption (roots, foliage) and movement of biostimulant components to the action sites (cytoplasm, chloroplasts, mitochondria, nucleus), followed by the regulation of plant hormones. These hormones act as primary signaling molecules that trigger the expression of antioxidant defense genes, activation of secondary metabolism, osmotic regulation, hormonal signaling, as well as other physiological and morphological changes in plants [91].

3.4.2. Impact of Environmental and Treatment Factors at Mid-Stages

Similar to early stages, E1 had the most favorable conditions for G20. This genotype was at an earlier developmental stage compared to the others when surface fertilization occurred, with nitrogen (N) needs being high and the need for N uptake being immediate. Although nutrient and water uptake are independent processes, the connections between nutrients and water are adjacent and complex, as soil water availability affects the migration, transformation, and accumulation processes of crops [92–94].

Environment 2 and E4 were the most challenging for both genotypes in terms of yield and quality traits, respectively. Regarding quantitative traits, they were likely affected by the high temperatures that prevailed during the anthesis stages (Figure 1B,D), when pollination of the grains occurs. As a result, this stage may have lasted for a shorter duration, leading to a reduction in the number of grains per spike and lower yields (Table 4) [95–97]. In E4, the genotypes lodged (Lodging) due to intense rainfall in the first decade of May (33.9 mm), just before harvest. This contributed both to a drop in yields and, primarily, to a reduction in grain size (Table 5) [98].

At mid-stages, the results of this study highlight the impact of biostimulant treatments (UI+B and U+B) on the improvement of traits in G20, during their growth. The application of biostimulants at the beginning of stem elongation (Z30) and surface fertilization at mid-stem elongation (Z32) appears to have a significant positive effect on both quantitative and qualitative characteristics. This genotype-treatment interaction emphasizes the need to tailor treatment strategies to specific stages of application [99,100]. The UI+B treatment was more effective in improving yield and TGW for G20, but in a few cases, it showed statistically significant differences from U+B. The impact of urease inhibitors on yield is not always detectable, with Murray et al. [101] finding no differences in yield quantity and quality between fertilizer formulations. Qianqian Li et al. [73], report that NBPT decreased NH_3 volatilization but did not significantly increase wheat grain yields. No specific study has determined the most efficient stages for applying urease inhibitor fertilizers to cereals. However, in barley, the 'critical period' for N concentration occurs immediately before heading, when the most relevant yield components are determined [102]. By this stage, N uptake by plants may be similar between the two forms of fertilizer applied in surface fertilization. The absence of major changes in these traits suggests that, while surface fertilization and biostimulants can influence specific aspects of plant development, other factors such as timing of fertilization [26] and environmental conditions [25,94] may have played a more dominant role in determining these characteristics.

Moreover, fast-developing genotypes like G20, later application of treatments may not provide as much benefit in terms of yield enhancement compared to slower-developing varieties. Sun et al. [103] note that fertilization timing significantly affected plant height, aboveground biomass, grain

yield, and leaf area index in wheat in China. The same results were observed by Kostić et al. [104], who concluded that the timing of N application had a highly significant effect on grain yield, biomass, and NUE traits. The type of fertilizer also plays a significant role in the effectiveness of surface fertilization. Fertilizers with urease inhibitors release N at a lower rate than those with plain urea [105,106], and thus, applying them at late stages of malting barley cultivation does not have a positive effect due to the lack of available N for crops. Resources that limit crop growth during stem elongation are allocated to the survival of tillers (improving spikelets per square meter), meaning there will be fewer resources available per spike, leading to a trade-off in grains/spike and vice versa [107].

In these experiments, the application of *Ascophyllum* nodosum extract between tillering and stem elongation showed positive effects on quantitative traits. Similarly, Hřivna et al. [82], report that the best phases of vegetation growth for biostimulant applications were during barley tillering and elongation. This application improved crop growth while reducing the amount of N required to achieve similar or higher yields of barley yield components compared to high-N treatments. The positive effects are linked to phytohormones such as auxins, cytokinins, abscisic acid, and substances with similar effects [66,82,83,90]. Cytokinins play a critical role in promoting cell division and modifying apical dominance [55], while auxins improve cell elongation and promote stem growth [108]. However, to date, no research has been published that examines the differences in the effects of ANE between stages with such high precision across stages in cereals.

The results for maltable fraction showed no statistically significant differences between the treatments (U, U+B, UI, UI+B) for G20, with all treatments meeting the industry-accepted limits across all environments. This suggests that while different treatments may enhance other parameters, they do not negatively affect maltable grain quality. This finding is likely due to the fact that the genotypes tested in this study have been evaluated over several years and selected among many others, often meeting the desired brewing quality characteristics [109].

For the G20, the UI+B treatment was particularly effective under favorable conditions (E1), leading to a 19.31% increase in grain size greater than 2.8 mm and a 6.06% increase in retention fraction compared to the control. The effects of biostimulants on plant nutrition and defense under stress, according to Akhter et al. [110], Lephatsi et al. [111], and Nephali et al. [91], are the result of their ability to activate signaling mechanisms for external danger detection, which were integrated into the plant genome during evolution as memory genes for responding to environmental stress signals.

Regarding GPC, G20 showed relative stability across all treatments (U, U+B, UI, UI+B). However, only the UI+B treatment met the acceptable GPC limits across all environments. This indicates that UI+B not only improves quality traits related to grain plumpness but also ensures stability in protein content, which is crucial for both quality assurance and market acceptance. This genotype may be more resilient to climatic variations, making it a suitable candidate for stable malting barley production. In recent studies, genotypes with heavier and plumper grains under high temperatures exhibited a higher grain filling rate, longer grain-filling duration, extended retention of green leaf area, increased water-soluble carbohydrate content, taller stature, smaller leaf size, greater grain weight/plumpness potential, and earlier flowering [112,113].

The PCA results highlight the consistent influence of combined biostimulant and fertilization treatments, particularly UI+B, on early plant performance. The distinct separation of the control treatment along PC1 in both application stages suggests that PCA successfully captured the impact of inputs on multiple early growth traits. Interestingly, treatment effects were more cohesive at the earlier application, supporting the hypothesis that early intervention can have a stronger and more uniform impact on plant vigor. This aligns with findings from previous studies, which demonstrated that early-stage application of seaweed extracts or humic substances can promote tillering, improve early biomass accumulation, and enhance root development [44,114].

The increased dispersion among treatments in the second application stage may indicate greater interaction between treatment effects and the physiological status of the plants or environmental

variation, which often becomes more pronounced as development progresses. PCA thus proved to be a valuable exploratory tool for assessing the overall structure of treatment effects on early development, revealing both shared and stage-specific responses. These insights may guide future decisions on the optimal timing and combination of biostimulant inputs in cereal production systems.

4. Discussion

The results of the present study provide a detailed investigation of the impact of climatic conditions, treatment's stage (represented by the different genotypes), and fertilization treatments, on both yield and quality traits of malting barley. Five treatments were applied: simple urea (U), U with biostimulant *Ascophyllum nodosum* extract (ANE) (U+B), urea with urease inhibitor (UI), UI combined with biostimulant ANE (UI+B), and control, without surface fertilization and ANE, across four environments, on two genotypes (G20 and G45). The findings underscore the complex interplay between environmental factors, genotypic characteristics, and agronomic practices in determining barley productivity and quality.

4.1. Impact of Environmental and Treatment Factors at Early Stages

Grain yield, in combination with key yield components such as aboveground biomass, harvest index (HI), spikelets m², and grains per spike, were used to measure and interpret the results [57]. In malting barley, aside from achieving high yields, specific quality criteria must be met regarding grain protein content (GPC) and grain size to satisfy industrial requirements. In terms of quality traits, the results of this study emphasize the importance of selecting the right genotype and fertilization strategy to meet the specific needs of the malting industry. Grain protein content (GPC) in malting barley is a critical quality trait, with an optimal range between 9.5% and 11.5%, while the retention fraction (proportion of grains larger than 2.5 mm) must exceed 90% [9].

In E1, more favorable climatic conditions were observed for G45, which exhibited the highest values for both quantitative and qualitative traits. The amount of rainfall (291.4 mm) and its distribution, combined with milder temperatures (Tmean = 11.17°C) during the growing season, and the low temperatures during the grain filling stage, made E1 the most ideal for malting barley cultivation, findings that align with existing literature [58,59].

Less favorable conditions prevailed in E4, where G45 had the lowest values for productive and quality traits. Although temperatures during the critical stages of growth were mild and rainfall (334.4 mm) was adequate, it is likely that the genotypes suffered from lodging after the rainfall (33.9 mm) that occurred in the first ten days of May (Figure 1D). The high-water availability during the vegetative stages of the crop led to an increase of the plants' height due to the rate of cell division [60]. In contrast, in Environment 2, the lower rainfall during the vegetative stages, resulted shorter plants more resistant to lodging, especially during the greater rainfall in May in this environment. However, in E2 reduction in grain size was observed which may be attributed to drought stress during late grain filling, factor which restricts carbohydrate incorporation in the grain, leading to a decrease in grain size despite consistent total nitrogen content [19]. The sensitivity of barley cultivars to both temperature extremes and the timing of precipitation with impact on yield components and quality characteristics have also been stated from other researchers [61–63].

The application of the biostimulant ANE and surface fertilization at early stages, had a pronounced effect on both quantitative and qualitative traits, particularly on G45. Among the different treatments, UI+B showed the most consistent and significant improvements on traits like aboveground biomass, yield, and thousand-grain weight (TGW) across all environments. This occurred, even though the UI+B treated at the lower dose (15 kg N/ha), compared the simple urea treatments (U and U+B). The combination of urea with urease inhibitors (NBPT) and the biostimulant ANE has been extensively studied, with positive effects observed in crops [64–67]. However, little is known about how ANEs influence nitrogen (N) uptake and assimilation mechanisms under reduced N conditions [65,68]. The integration of biostimulants with current farming practices to enhance NUE may present challenges (e.g., method of application, timing, rates) in some production systems

[65,69,70]. Nevertheless, the addition of ANE played a significant role in positively influencing plant growth, particularly by stimulating root development and nutrient uptake, likely facilitating the observed improvements in yield. Specifically, field experiments showed that foliar application of engineered ANE biostimulants maintained or increased grain yield in barley grown with 25% less N fertilizer [65]. Maignan et al., [71], report that ANE significantly improved grain yield (+3.59 tn/ha), total grain N, and NUE. ANE application improved growth during tillering and stem elongation increased the number of fertile tillers, grain number per m², and TGW [69,71,72].

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The aforementioned results are likely a consequence of ANE's ability to generally stimulate root growth and increase the efficiency of soil nutrient absorption and utilization [82,83]. An increase in nutrient uptake efficiency was also observed in cereals (H. vulgare, Triticum aestivum/durum, Zea mays). In malting barley, a larger root system increases starch content and malt extract during the dry season [82,84]. In contrast, a weak root system under dry conditions leads to low grain yield and a significant deterioration in quality [82,84]. Moreover, Goettl et al. [85] report that N plays a role in grain size. However, the literature supports the notion that GPC is inversely associated with grain size [16,19]. This is because starch accumulation appears to be more sensitive to post-anthesis stress than nitrogen accumulation [19,86]. In this study, this claim was not confirmed, as climate conditions and genotype played a more important role [87–89]. It is worth noting that the addition of ANE improved plant tolerance in environments where stressful growing conditions prevailed, something which have been stated by other researchers [66,82,83,90]. The effects of biostimulants on plant defense and adaptation to abiotic stresses include, initially, the absorption (roots, foliage) and movement of biostimulant components to the action sites (cytoplasm, chloroplasts, mitochondria, nucleus), followed by the regulation of plant hormones. These hormones act as primary signaling molecules that trigger the expression of antioxidant defense genes, activation of secondary metabolism, osmotic regulation, hormonal signaling, as well as other physiological and morphological changes in plants [91].

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Similar to early stages, E1 had the most favorable conditions for G20. This genotype was at an earlier developmental stage compared to the others when surface fertilization occurred, with nitrogen (N) needs being high and the need for N uptake being immediate. Although nutrient and water uptake are independent processes, the connections between nutrients and water are adjacent and complex, as soil water availability affects the migration, transformation, and accumulation processes of crops [92–94].

Environment 2 and E4 were the most challenging for both genotypes in terms of yield and quality traits, respectively. Regarding quantitative traits, they were likely affected by the high temperatures that prevailed during the anthesis stages (Figure 1B,D), when pollination of the grains occurs. As a result, this stage may have lasted for a shorter duration, leading to a reduction in the number of grains per spike and lower yields (Table 4) [95–97]. In E4, the genotypes lodged (Lodging) due to intense rainfall in the first decade of May (33.9 mm), just before harvest. This contributed both to a drop in yields and, primarily, to a reduction in grain size (Table 5) [98].

At mid-stages, the results of this study highlight the impact of biostimulant treatments (UI+B and U+B) on the improvement of traits in G20, during their growth. The application of biostimulants at the beginning of stem elongation (Z30) and surface fertilization at mid-stem elongation (Z32) appears to have a significant positive effect on both quantitative and qualitative characteristics. This genotype-treatment interaction emphasizes the need to tailor treatment strategies to specific stages of application [99,100]. The UI+B treatment was more effective in improving yield and TGW for G20, but in a few cases, it showed statistically significant differences from U+B. The impact of urease inhibitors on yield is not always detectable, with Murray et al. [101] finding no differences in yield quantity and quality between fertilizer formulations. Qianqian Li et al. [73], report that NBPT decreased NH_3 volatilization but did not significantly increase wheat grain yields. No specific study has determined the most efficient stages for applying urease inhibitor fertilizers to cereals. However, in barley, the 'critical period' for N concentration occurs immediately before heading, when the most relevant yield components are determined [102]. By this stage, N uptake by plants may be similar between the two forms of fertilizer applied in surface fertilization. The absence of major changes in these traits suggests that, while surface fertilization and biostimulants can influence specific aspects of plant development, other factors such as timing of fertilization [26] and environmental conditions [25,94] may have played a more dominant role in determining these characteristics.

Moreover, fast-developing genotypes like G20, later application of treatments may not provide as much benefit in terms of yield enhancement compared to slower-developing varieties. Sun et al. [103] note that fertilization timing significantly affected plant height, aboveground biomass, grain yield, and leaf area index in wheat in China. The same results were observed by Kostić et al. [104], who concluded that the timing of N application had a highly significant effect on grain yield, biomass, and NUE traits. The type of fertilizer also plays a significant role in the effectiveness of surface fertilization. Fertilizers with urease inhibitors release N at a lower rate than those with plain urea [105,106], and thus, applying them at late stages of malting barley cultivation does not have a positive effect due to the lack of available N for crops. Resources that limit crop growth during stem elongation are allocated to the survival of tillers (improving spikelets per square meter), meaning there will be fewer resources available per spike, leading to a trade-off in grains/spike and vice versa [107].

In these experiments, the application of *Ascophyllum* nodosum extract between tillering and stem elongation showed positive effects on quantitative traits. Similarly, Hřivna et al. [82], report that the best phases of vegetation growth for biostimulant applications were during barley tillering and elongation. This application improved crop growth while reducing the amount of N required to achieve similar or higher yields of barley yield components compared to high-N treatments. The positive effects are linked to phytohormones such as auxins, cytokinins, abscisic acid, and substances with similar effects [66,82,83,90]. Cytokinins play a critical role in promoting cell division and modifying apical dominance [55], while auxins improve cell elongation and promote stem growth

[108]. However, to date, no research has been published that examines the differences in the effects of ANE between stages with such high precision across stages in cereals.

The results for maltable fraction showed no statistically significant differences between the treatments (U, U+B, UI, UI+B) for G20, with all treatments meeting the industry-accepted limits across all environments. This suggests that while different treatments may enhance other parameters, they do not negatively affect maltable grain quality. This finding is likely due to the fact that the genotypes tested in this study have been evaluated over several years and selected among many others, often meeting the desired brewing quality characteristics [109].

For the G20, the UI+B treatment was particularly effective under favorable conditions (E1), leading to a 19.31% increase in grain size greater than 2.8 mm and a 6.06% increase in retention fraction compared to the control. The effects of biostimulants on plant nutrition and defense under stress, according to Akhter et al. [110], Lephatsi et al. [111], and Nephali et al. [91], are the result of their ability to activate signaling mechanisms for external danger detection, which were integrated into the plant genome during evolution as memory genes for responding to environmental stress signals.

Regarding GPC, G20 showed relative stability across all treatments (U, U+B, UI, UI+B). However, only the UI+B treatment met the acceptable GPC limits across all environments. This indicates that UI+B not only improves quality traits related to grain plumpness but also ensures stability in protein content, which is crucial for both quality assurance and market acceptance. This genotype may be more resilient to climatic variations, making it a suitable candidate for stable malting barley production. In recent studies, genotypes with heavier and plumper grains under high temperatures exhibited a higher grain filling rate, longer grain-filling duration, extended retention of green leaf area, increased water-soluble carbohydrate content, taller stature, smaller leaf size, greater grain weight/plumpness potential, and earlier flowering [112,113].

The PCA results highlight the consistent influence of combined biostimulant and fertilization treatments, particularly UI+B, on early plant performance. The distinct separation of the control treatment along PC1 in both application stages suggests that PCA successfully captured the impact of inputs on multiple early growth traits. Interestingly, treatment effects were more cohesive at the earlier application, supporting the hypothesis that early intervention can have a stronger and more uniform impact on plant vigor. This aligns with findings from previous studies, which demonstrated that early-stage application of seaweed extracts or humic substances can promote tillering, improve early biomass accumulation, and enhance root development [44,114].

The increased dispersion among treatments in the second application stage may indicate greater interaction between treatment effects and the physiological status of the plants or environmental variation, which often becomes more pronounced as development progresses. PCA thus proved to be a valuable exploratory tool for assessing the overall structure of treatment effects on early development, revealing both shared and stage-specific responses. These insights may guide future decisions on the optimal timing and combination of biostimulant inputs in cereal production systems.

5. Conclusions

This study highlights the critical influence of environmental conditions, genotype characteristics, and nitrogen management strategies on the productivity and quality of malting barley. The combination of urease inhibitor-treated urea and a seaweed-based biostimulant (UI+B) consistently improved grain yield and grain size, while maintaining grain protein concentration (GPC) within industry-acceptable limits across diverse environments. At early application stages (Z24–Z30), the UI+B treatment was particularly effective for G45, significantly enhancing both yield and qualitative traits even under challenging climatic conditions. Conversely, mid-stage applications (Z30–33) demonstrated a genotype-dependent effect, with UI+B benefiting G20. Principal Component Analysis (PCA) underscored the importance of treatment timing and genotype adaptability in differentiating multivariate performance patterns. The PCA effectively revealed distinct separation between the control and treated samples, with UI+B-treated plots consistently clustering based on

favorable agronomic and quality traits. While biostimulant application consistently improved grain plumpness, treatment efficacy varied depending on the phenological stage and environmental stress conditions. Overall, the integration of biostimulants with enhanced-efficiency fertilizers offers a promising agronomic strategy for improving barley yield and malting quality, while also supporting more sustainable nitrogen management. Future research should aim to refine biostimulant application protocols, deepen understanding of genotype-specific responses, and validate these findings under a broader range of environmental conditions to further optimize malting barley production systems.

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Abbreviations

The following abbreviations are used in this manuscript:

GPC	Grain Protein Content
UI	Urease Inhibitor
UI+B	Urea with Urease Inhibitor and Biostimulan
U	Urea
U+B	Urea with Biostimulant
ANE	*Ascophyllum nodosum* Extract
TGW	Thousand Grain Weight
RCB	Randomized Complete Block design
PCA	Principal Component Analysis
LSD	Least Significant Difference
E1, E2, E3, E4	Experimental Environments (different locations/seasons in Thessaly)

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