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Enhancing Organic Selenium Forms in Alfalfa Forage Through Inorganic Selenium Foliar Application: Insights from Laboratory and Field Studies Using X-Ray Spectroscopy

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Abstract: Selenium (Se) is an essential micronutrient, yet its deficiency remains a global concern. This study investigates the biofortification of alfalfa (*Medicago sativa* cv. ProINTA Super Monarca GR9) via foliar Se application to enhance Se accumulation and transformation into bioavailable organic forms. A controlled environment experiment in a plant growth chamber and a one-season open-field trial (January 2023, Argentina) were conducted. Treatments included sodium selenate (Se(VI)), sodium selenite (Se(IV)), and a 1:1 mixture, applied at 45 and 90 g Se ha^{-1}, with and without the biostimulant BIOFORGE®. Treated plants exhibited increased Se content, correlating with the applied doses. X-ray absorption spectroscopy (XAS) confirmed that most inorganic Se was transformed into organic Se forms, with Se(IV) treatments yielding the highest concentrations of organic Se species such as selenocysteine (SeCys) and selenomethionine (SeMet). Open-field trials showed a complete conversion of Se, though total Se accumulation was lower than in controlled conditions. Se treatments did not affect forage quality or biomass production. The biostimulant slightly reduced Se uptake but did not compromise biofortification. These results highlight Se(IV) as the optimal treatment for alfalfa biofortification, presenting a sustainable strategy to enhance dietary Se intake through functional foods.

Keywords: biofortification; selenate; selenite; direct speciation; synchrotron

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1. Introduction

Selenium (Se) is an essential micronutrient that plays a pivotal role in supporting proper physiological functions in both animals and humans. It is indispensable for the synthesis and activity of more than 25 selenoproteins, including glutathione peroxidase, thioredoxin reductase, and selenoprotein P. These selenoproteins are crucial for antioxidant defense, hormonal regulation, male fertility, resistance to viral infections, and cancer prevention [1]. Among these, selenocysteine, a unique amino acid encoded by the UGA codon, is central to the enzymatic activity of selenoproteins [2]. Selenium's synergistic interaction with vitamin E further enhances its antioxidant efficacy, underscoring its importance in mitigating oxidative stress and promoting overall health [3]. Through these mechanisms, Se is indispensable for diverse physiological functions, making its adequate intake a key factor in promoting health and preventing disease.

Although this key role of Se in human health and disease has been extensively highlighted during the last decades [4], Se deficiency is still a global issue that predominantly affects Asia and Europe. Indeed, it is estimated that approximately 1 billion people worldwide has Se deficiency [5]. Globally, approximately one billion people are affected by Se deficiency, which has been associated with increased risks of cardiovascular disease, weakened immune function, and neurodegenerative disorders. In response to this challenge, strategies to enhance Se intake through dietary sources have gained attention, particularly through biofortification—an agricultural approach aimed at increasing the concentration of bioavailable nutrients in edible plants.

To improve human Se intake, some studies have taken the approach of research the supplementation of dairy cows with selenium to determine whether the inorganic selenium content in milk increased. For example, in Hungary (that belongs to the selenium-deficient regions in Europe), an experiment with daily supplementation of 1–6 mg organic Se to the feed of dairy cows increases the selenium content of milk from the value of 18 μg kg^{-1} to 94 μg kg^{-1} in 8 weeks, decreasing again to the initial value in 6 weeks after stopping the supplementation [6].

Furthermore, the consumption of Se-enriched food has increased moderately in recent years as the populace has become more cognizant of the issue. In that respect, Se biofortification of plants has emerged as a sustainable, cost-efficient, and safe strategy to combat Se deficiency with plant-based agricultural products [2,7–9].

In plants, Se enhances photosynthetic rates, growth, and development while promoting the accumulation of not only Se but also essential minerals such as iron (Fe) and zinc (Zn) [10,11]. Studies on *Brassicaceae* species have also demonstrated increased concentrations of Se and other nutrients following Se application [12]. Furthermore, Abdalla et al. [12] showed that Se application can increase the synthesis of organic Se species in crops, such as selenomethionine (SeMet), selenocysteine (SeCys), and methylselenocysteine (MeSeCys); these plant-derived compounds have anticarcinogenic properties in humans [5]. However, SeCys and SeMet can be incorporated into plant's proteins, replacing cysteine (Cys) and methionine (Met), respectively. Thus, excessive Se is generally harmful to plant health, which could in turn decrease the absorption of Se and reduce growth rate [5].

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops worldwide, valued not only for its high nutritional content, which makes it a key resource for dairy farming, but also for its positive contributions to health and environmental sustainability [13]. Alfalfa is a rich source of protein (17–20% crude protein), carbohydrates, fiber, and essential vitamins (A, B, C, E, and K), as well as minerals such as calcium, phosphorus, copper, and potassium, which are vital for the livestock and dairy industries [14]. Globally, alfalfa is cultivated on approximately 30 million hectares, with major production areas in North and South America, Europe, and Asia [15]. In Argentina, for instance, the alfalfa-growing area is

estimated at 3.4 million hectares, with approximately 60% dedicated to pure alfalfa fields for dairy farming and hay production [16].

The application of Se in plants, particularly through foliar application of Se salts such as sodium selenate and sodium selenite, has been demonstrated to be an effective strategy for biofortification. Foliar selenate amendments have consistently increased forage Se concentrations in a dose-dependent manner, regardless of the forage type, including orchard grass, grass–clover mixtures, and alfalfa [17,18]. Foliar fertilization with Se, Zn, and Cu could be an important agrotechnical measure for increasing these elements in alfalfa grown in slightly alkaline soil under rainfed conditions [19]. Other studies reported that foliar application of sodium selenate in alfalfa increased selenium content in plants up to 112.5% and 182%, respectively, compared with the control treatment in non-calcareous and calcareous soils [20].

Moreover, foliar applications of these mineral forms can improve physiological parameters in plants, including net photosynthesis rate and water use efficiency, as observed in crops like broccoli [21]. Furthermore, recent studies underscore the potential of Se-biofortified alfalfa microgreens as functional foods. These microgreens are highly nutritious, offering essential vitamins, minerals, and enzymes, and represent an innovative strategy to enhance Se intake in human diets [22].

Considering the ability of some plants to accumulate and transform Se into bioactive compounds, it follows that the use of Se-enriched plant species can have important implications in human nutrition and health [9]. However, bioavailability but also toxicity of Se is closely correlated to the chemical form ingested; in this order, organic Se species have shown more bioavailability than inorganic species for humans [23,24]. Therefore, Se species characterization in food and food supplements has become necessary. Thus, chemical speciation of Se in the biofortified alfalfa are also studied to better understand the mechanisms of Se absorption and metabolization [25]. Usually, chromatographic analytical techniques coupled with mass spectrometers, such as HPLC-ICP-MS, are used for determining the speciation of Se indirectly. However, several pretreatment steps are required to extract and solubilize the Se species, affecting the structure of the original compounds or producing incomplete recoveries of them [26]. To overcome these limitations, direct speciation techniques, like X-ray absorption spectroscopy (XAS), offer a solution that allows speciation analysis of Se in solid form without the need for any extraction or pretreatment steps [27].

Consequently, the objective of this research was to evaluate the effects of foliar Se application on Se accumulation and speciation, mineral composition, forage quality, and yield in alfalfa plants. It was hypothesized that foliar application of selenium in alfalfa increases the amount of selenium in the plant and the amount of this element in the organic form. Different biofortification treatments were tested, involving the application of Se in the form of selenate, selenite, or a combination of both, at two doses (45 or 90 g ha^{-1}), with and without the addition of a plant biostimulant. These treatments were initially evaluated at the laboratory scale to identify the most suitable options, which were subsequently applied in open-field trials.

Given the critical role of selenium (Se) in human and animal nutrition, along with the agronomic and nutritional significance of alfalfa, this study aimed to determine the most effective Se salt for optimizing Se bioavailability, mineral composition, forage quality, and crop yield. The findings of this work contribute to an increase in the selenium content of alfalfa plants, which will enable cows to transform this food into selenium-enriched milk. This, in turn, will contribute to a higher Se intake from dairy products.

2. Materials and Methods

2.1. Se-Biofortified Alfalfa at Laboratory Scale

Five seeds of alfalfa (*Medicago sativa* cv. ProINTA Super Monarca GR9) were sown in plastic pots (10 cm in diameter × 100 cm in height) containing sterile sand as the substrate. The pots were placed in a plant growth chamber at the Laboratory of Research in Physiology and Plant Molecular Biology (LIFiBVe, Faculty of Agricultural Sciences, National University of Litoral, ICiAgro CONICET, Santa Fe, Argentina), irrigated periodically with 50% Hoagland solution, and maintained at 25 ± 3 °C in complete darkness. After five days, three homogeneous seedlings per pot were selected for each experimental unit. The culture was conducted at the same constant temperature with 60–70% relative humidity. The mean irradiance was 234 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation) under a 16 h light/8 h dark photoperiod.

After 25 days, the first cut was made at 10 cm above the plant crown. Ten days later, at the peak of vegetative growth, the treatments were applied foliarly using a manual sprayer. Photosynthetic parameters and the chlorophyll index were measured 10 days after treatment application. Finally, 15 days post-treatment, the plants were harvested for analysis of growth parameters, including branch number, plant height, and fresh and dry weights of shoots and roots, as well as forage quality. The shoots (stems and leaves) were then separated, lyophilized, ground, and stored at −20 °C for further analysis.

2.2. Application of Se Foliar Treatments

Foliar Se applications were performed using selenite (Se(IV), Na_2SeO_3) (Sigma-Aldrich, Berlin, Germany), selenate (Se(VI), Na_2SeO_4) (Sigma-Aldrich, Berlin, Germany), and a 1:1 mixture of both (Se(Mix)), at rates of 45 and 90 g Se ha^{-1} [28,29]. This corresponded to an applied mass of 135 ng of Se(IV) and 147 ng of Se(VI) per pot for the 45 g Se ha^{-1} treatment, and 270 ng of Se(IV) and 294 ng of Se(VI) per pot for the 90 g Se ha^{-1} treatment. A 0.03% solution of Lauryl Alcohol Ethoxylate 7 Mole (Smart Bio SA, Santa Fe, Argentina) was added as an adjuvant to enhance the efficacy of foliar spraying. Applications were conducted with (+BIO) and without (-BIO) the co-application of a plant biostimulant (BIOFORGE®) (STOLLER Company, Córdoba, Argentina) at a dose of 600 mL ha^{-1} to mitigate potential Se toxicity. Distilled water with the adjuvant was used for treatments without Se (CONTROL). For applications, pots were individually transferred to a chamber separate from the cultivation area and physically isolated with a plastic barrier to prevent cross-contamination during spraying. Then, pots were divided into four subplots according to treatment: CONTROL, Se(IV), Se(VI), or mixture applied. Each subplot contained twenty experimental units, and pots were arranged in a completely randomized design. Control plants were grown under identical conditions to Se-treated plants.

2.3. Gas Exchange Measurements and Chlorophyll Index Determination

The net photosynthetic rate (Pn), transpiration rate (TRN) and stomatal conductance (gs) of the upper mature leaves were measured using a CIRAS-2 portable photosynthesis system (PP Systems, Amesbury, MA, USA) under conditions of 500 ppm CO_2 and 900 μmol m^{-2} s^{-1} PAR to ensure consistency and eliminate the influence of diurnal variations. The measurements were taken once the cuvette's leaf chamber of the CIRAS-2 equipment stabilized. This standardization protocol ensured that all measurements were comparable and reflected the plant's response to the controlled environment.

The chlorophyll index was determined on three leaves per plant, and the values were averaged using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Osaka, Japan) during photosynthesis measurement [30].

2.4. Forage Quality Assessment

Chemical parameters were analyzed according to standardized procedures [31]. Dried matter (DM) was determined by drying at 65 °C; crude protein (CP) was calculated as Total Nitrogen × 6.25 using the Kjeldahl method. Neutral detergent fiber (NDF) was measured with added heat stable α-amylase and sodium sulphite, while acid detergent fiber (ADF) and acid detergent lignin (ADL) were measured using an ANKOM fiber analyzer (Model 220, ANKOM Tecnology, Macedon, NY, USA). Ether extract (EE) was determined using the Soxhlet extraction method, and ash content was measured by heating at 600 °C for 2 h [32].

2.5. Open-Field Se-Biofortification of Alfalfa

Based on the results previously obtained at the laboratory scale, two doses of Se(IV) (45 g ha^{-1} and 90 g ha^{-1}) were selected for the open-field trial. These were applied as an inorganic Se salt with and without the same biostimulant, and using the same adjuvant as in the laboratory assay. The evaluation took place in January 2023. A second-year alfalfa lot (cv. ProINTA Super Monarca GR9) was used, located in a 24 m × 40 m area divided into 24 plots of 6 m × 4 m each. The experiment was conducted in the experimental agronomy field of INTA EEA Rafaela, Santa Fe, Argentina (31.35 S, 61.49 W, altitude 100 m).

The soil used was a Mollisol, specifically classified as a Typic Argiudoll. These soils are prevalent in the Pampas region of Argentina, particularly in the provinces of Buenos Aires, Santa Fe, Entre Ríos, and Córdoba. They are characterized by a deep profile, often reaching up to 120 cm, with a surface horizon containing approximately 3% organic matter. The argillic horizon typically has a clay content ranging from 30% to 50%. In deeper layers, such as the BC and C horizons, calcium carbonate nodules may be present [33]. Before the assay, a soil chemical analysis was performed on a composite sample using a stainless-steel tube sampler to a depth of 0.20 m (Table S1).

The evaluated treatments included control groups without Se application, both with and without a biostimulant and the application of Se(IV) at doses of 45 and 90 g ha^{-1}, also with and without a biostimulant. The experimental design used was one factor measured at 6 levels, with 4 replicates and 4 samples taken per replicate (quadruplicates). The pasture was cut at a height of 10 cm above the plants' crown prior to application. Foliar application of inorganic Se was carried out when plants had 2–3 nodes, and the pasture was sampled for quality evaluation at the 7–8 node stage. The dose applied was 80 L ha^{-1} (45 g ha^{-1} or 90 g ha^{-1} Se(IV) + 600 mL ha^{-1} of biostimulant + 0.15% adjuvant with groundwater). Spring–summer alfalfa pastures were sown on April 2020 with a planting density of 12 kg ha^{-1}. The first harvest was conducted on January 4, 2023, at a height of 10 cm from the crown, followed by a foliar Se application on January 16, 2023 when the plants were at the 2–3 node stage. A second harvest, aimed at evaluating physicochemical parameters, was performed on January 30, 2023 when the plants were at the 7–8 node phenological stages. During the experimental period (4–30 January 2023), the average temperature was 30.6 °C, with a maximum of 36.4 °C, a minimum of 19.1 °C, and rainfall of 70.9 mm. Compared to the historical average (1930 to 2019), the recorded temperatures were higher (mean: 26.3 °C, maximum: 31.8 °C, and minimum: 18.3 °C), while rainfall was lower (121.4 mm historical average from 1930 to 2022 [34].

2.6. Determination of Total Selenium, Macro- and Micronutrient Concentration

Total concentrations of Se, micronutrients (B, Mn, Fe, Cu, Zn, Mo, Ni), and macronutrients (Mg, P, K, Ca) were analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Prior to analysis, the samples (alfalfa shoots) were acid digested with 7 mL of 65% nitric acid and 3 mL of H_2O_2. The digestions were carried out in an analytical microwave

(Mars 5, CEM Corporation, Matthews, NC, USA) with a temperature and pressure gradient reaching up to 180 °C and 1.9 atm for 45 min.

After digestions, the samples were filtrated and diluted and concentrations of ^{11}B, ^{55}Mn, ^{56}Fe, ^{64}Zn, ^{65}Cu, ^{78}Se, ^{98}Mo, ^{24}Mg, ^{31}P, ^{39}K and ^{44}Ca were measured by ICP-MS (X Series 2, Thermo Elemental, Loughborough, UK). Macronutrient S was measured by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Agilent 5900, Agilent Technologies, Santa Clara, CA, USA).

2.7. Selenium Speciation Analysis by XAS

X-ray absorption near edge structure (XANES) spectra were collected at Se K-edge in CLAESS beamline at ALBA Synchrotron Light Facility, Cerdanyola del Vallès, Spain [35]. The synchrotron radiation emitted by a wiggler source was monochromatized using a double crystal Si(311) monochromator. Higher harmonics were rejected by choosing proper angles and coatings of the collimating and focusing mirrors. The x-ray beam was unfocused up to around 2 × 1 mm^2 and the measurements acquired at the liquid nitrogen temperature to minimize eventual radiation damage.

Approximately 30 mg of lyophilized and ground alfalfa shoots, from plants biofortified with Se, were homogenized and pressed into pellets (5 mm diameter). Each sample was prepared by mixing five replicates to account for biological variability.

The measurements were performed in the fluorescence mode due to the low concentration of Se in the plant samples using the multi-element Si drift detector with XSpress3 electronics. All the measurements were performed at liquid nitrogen temperature to avoid radiation damage of the samples. The spectra were collected on 3 spots of each pellet to account for eventual sample inhomogeneities and to reduce the local exposure of the sample to the X-rays. No radiation damage was detected and neither were inhomogeneities; thus, all the spectra measured on a pellet were merged to improve statistics.

Furthermore, reference samples of Se(IV), Se(VI), seleno-L-methionine, seleno-L-cystine, and Se-(Methyl)selenocysteine hydrochloride were measured for comparison. The references were prepared as aqueous solutions for comparison with the samples considering the possible changes between the structure of the compounds in the solid state and in the plants. The solutions were loaded into an in-house-designed liquid cell with a 2 mm transmission path and Kapton windows [36]. The references were measured in transmission mode. For energy calibration, the Se K-edge XANES spectrum of the elemental Se was used and the energy of the maximum of the first derivative was set to 12,658 eV.

Normalization of the XANES data using standard procedures was performed with the ATHENA program of the DEMETER software package version 0.9.26 [37].

XANES spectra were subjected to multivariate curve resolution-alternating least squares (MCR-ALS) unmixing using the MCR toolbox [38] running under Matlab version 8.6.0 (release 2015b) (The Mathworks inc, Natick, MA, USA) environment. In detail, Simplisma [39] was used to obtain an initial estimate for the spectra of the pure components, and then the ALS algorithm was operated using as constraints the non-negativity for spectra and concentration profiles and equality for the last points of the spectra (signal equal to 1 for the last 20 data points of the XANES profiles). Five components were found to be the best model complexity and correspond to Se(VI), Se(IV), SeCys, other organic Se, and SeMet/SeMetCys species.

2.8. Statistical Analysis

The experimental data were statistically analyzed using InfoStat statistical software version 2017 (Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina) [40]. The significance of the difference among the

mean values was determined by one-way analysis of variance (ANOVA) with subsequent multiple comparisons of means by the least significant difference (LSD) test. Correct application of ANOVA was checked by residual normal distribution (Shapiro–Wilks Test, QQ plots) and homoscedasticity (Levene test, residual plots). Differences at $p < 0.05$ were statistically significant. The data are expressed as the mean ± standard error (SE).

The relations between the agronomic parameters and the relative concentrations of the various Se species (as estimated by the MCR resolved components) were further examined by calculating the correlation coefficients between all possible pairs of variables. For a more immediate inspection, the obtained correlation matrix was graphically displayed in the form of a heat map, i.e., an image where color-coding is exploited to represent the different numerical values.

3. Results and Discussion

3.1. Se-Biofortified Alfalfa at Laboratory Scale

3.1.1. Physiological Parameters

The foliar application of Se significantly affected the physiological parameters of alfalfa plants, with notable improvements observed particularly under Se(IV) treatments, irrespective of the presence of the biostimulant (Figure 1, Table S2).

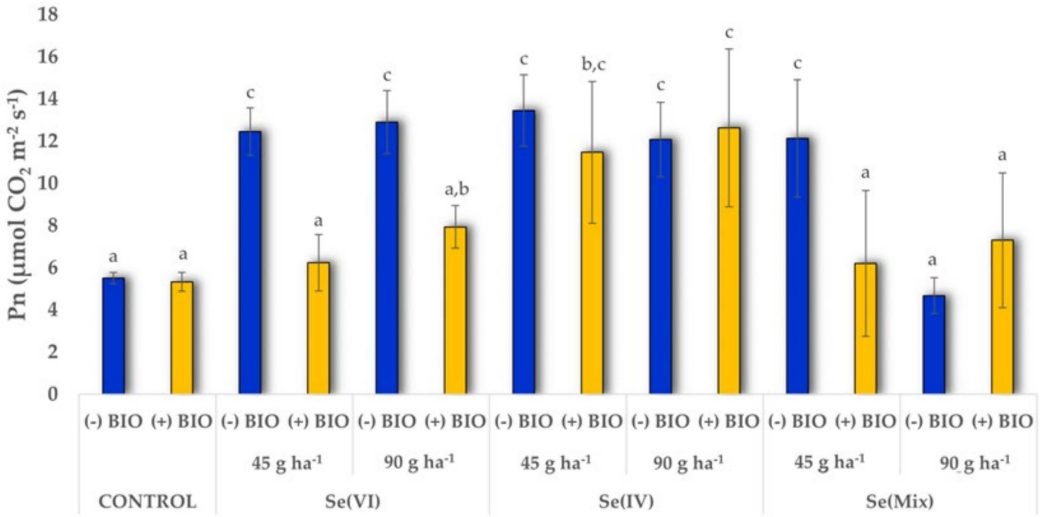

Figure 1. Effect of foliar selenium application on the leaf net photosynthetic rate (Pn) in alfalfa plants grown under different Se treatments in controlled conditions. -BIO: without biostimulant, +BIO: with biostimulant (BIOFORGE® 600 mL ha^{-1}), Se(Mix):Se(VI)/Se(IV) (1:1). Results are expressed as mean (n = 5) ± SE. Means not sharing any letter are significantly different by LSD test at $p < 0.05$ level of significance.

For instance, the net photosynthesis rate (Pn) increased by up to 144% in the Se(IV) +BIO treatment at 45 g ha^{-1} compared to the control. This enhancement was closely associated with higher stomatal conductance (gs) and transpiration rate (TRN), especially in Se(IV) treatments. Selenium supplementation generally enhances or has no effect on photosynthesis, depending on the plant species and application method [21,41,42]. For example, in rice (*Oryza sativa* L.), Se supplementation via root irrigation significantly improved growth, correlating with an increase in Pn. However, when Se was applied foliarly, photosynthesis remained unaffected, and biomass accumulation showed no significant changes [42].

On the other hand, although significant increases in gs and TRN were detected (Supplementary Table S2), a lower Pn was observed in the Se(Mix) 90 g ha^{-1} -BIO treatment compared to the other Se treatments without the biostimulant. It is well known that

while higher gs and TRN typically promote greater CO_2 uptake, potentially increasing Pn, this response may not occur if other factors, such as temperature, light, or CO_2 availability, become limiting [43]. If CO_2 levels are sufficient to saturate ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme in carbon fixation, further increases in gs do not enhance Pn, as the photosynthetic machinery is already operating at its maximum capacity for CO_2 assimilation [44]. This phenomenon, known as CO_2 saturation, may explain these findings. In this regard, it has been demonstrated that Rubisco expression was down-regulated in *Cardamine hupingshanensis* plants treated foliarly with high Se concentrations [45]. Therefore, a possible explanation for the results obtained here is that the observed increases in TRN and gs may have been offset by a reduction in Rubisco activity, leading to stable Pn despite the increased gas exchange. Thus, while Pn, TRN, and gs are generally correlated, the results suggest that the combined application of both Se species did not lead to significant changes in Pn at the 90 g ha^{-1} dose in alfalfa plants.

Additionally, SPAD values, which are widely recognized as reliable non-destructive indicators of chlorophyll concentration in plant tissue [46], showed no significant differences among Se treatments, regardless of the presence or absence of the biostimulant (Table S2). Consistent with our findings, Se supplementation has been reported to have no significant effect on chlorophyll content under control conditions in oilseed rape (*Brassica napus* [47] or wheat (*Triticum aestivum* L.) [48]. Similarly, stevia (*Stevia rebaudiana*) seedlings from seeds primed with sodium selenite exhibited chlorophyll levels comparable to control plants [49].

In contrast, Se effects on chlorophyll content vary depending on the species and concentrations used. For instance, a notable decrease in chlorophyll levels was observed in cucumber (*Cucumis sativus* L.) at Se concentrations lower than those causing growth inhibition [50]. Such reductions in photosynthetic pigments are considered a more sensitive indicator of Se phytotoxicity than declines in fresh weight or leaf area [18]. The lack of changes in SPAD values in alfalfa leaves suggests that the Se doses applied in this study were non-toxic and did not adversely affect photosynthetic pigment synthesis.

3.1.2. Effect of Foliar Selenium Application on Yield Parameters and Selenium Content in Alfalfa Plants Grown Under Controlled Conditions

Foliar Se treatments significantly influenced yield parameters in alfalfa plants, including the number of leaves and shoot fresh weight (FW). The highest values were observed with the application of Se(IV) +BIO at 45 g ha^{-1} (Table S3). Similarly, the application of Se(Mix) +BIO at 90 g ha^{-1} enhanced the number of branches. However, dry matter content decreased significantly, by approximately 18%, when 90 g ha^{-1} of Se(IV) was applied in combination with the biostimulant compared to the control without biostimulant. The reduction in dry matter accumulation induced by biostimulants can be attributed to their effects on the balance between plant growth and stress response processes. Biostimulants modulate physiological pathways to enhance stress tolerance, nutrient use efficiency, and overall plant health. However, this modulation may sometimes redirect energy and resources from biomass production toward stress adaptation or other physiological priorities, leading to a decrease in dry biomass accumulation under specific conditions [51,52].

Conversely, the application of Se resulted in a significant increase in Se concentration in the aerial parts of alfalfa plants compared to the control group (Figure 2). The Se content in alfalfa plants doubled with an application rate of 45 g ha^{-1} and quadrupled at 90 g ha^{-1}, irrespective of the Se species applied (Se(VI), Se(IV), or their combination). Interestingly, the co-application of Se with the biostimulant typically led to lower Se accumulation compared to treatments where Se was applied alone.

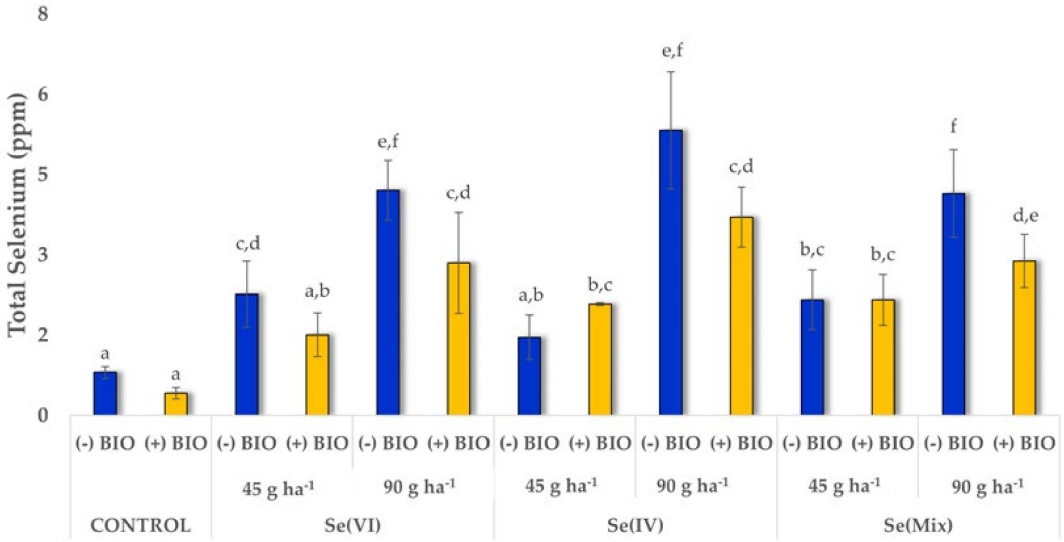

Figure 2. Total selenium concentration in the aerial part of alfalfa plants grown under different Se treatments in controlled conditions. -BIO: without biostimulant, +BIO: with biostimulant (BIOFORGE® 600 mL ha^{-1}), Se(Mix): Se(VI)/Se(IV) (1:1). Results are expressed as mean (n = 3) ± SE. Means not sharing any letter are significantly different by the LSD test at $p < 0.05$ level of significance.

These findings align with previous reports by Hall et al. [29] and Wang et al. [53], which demonstrated a linear increase in forage Se concentrations, from 2.06 to 4.15 mg kg^{-1} dry matter, when foliar Se(VI) application rates were doubled from 45 to 90 g ha^{-1}.

3.1.3. Effects of Selenium on Forage Quality in Alfalfa Plants Grown Under Controlled Conditions

The effect of foliar Se application was analyzed on various forage quality parameters (CP, NDF, ADF, and ADL) in alfalfa plants (Table S4). In this regard, no significant differences were observed in the analyzed parameters due to Se, either in the presence or absence of the biostimulant, compared to the controls.

Based on these results, it can be concluded that the foliar application of Se does not significantly affect the forage quality of alfalfa. This suggests that Se supplementation does not compromise key characteristics of forage quality, such as nutrient digestibility, intake potential, or palatability for livestock. Therefore, Se biofortification via foliar application remains a viable strategy without detrimental effects on the nutritional value or usability of the forage for cattle feeding.

3.1.4. Effects of Selenium on Mineral Elements in Alfalfa Plants Grown Under Controlled Conditions

The foliar application of Se influenced the mineral nutrient profile of alfalfa (Table S5). Among the micronutrients, the treatment with 90 g ha^{-1} of Se(IV) -BIO induced the most significant changes compared to the controls, reducing B content by 45% while increasing Mn by 54%, Fe by 47%, and Ni by 70%. Additionally, this treatment significantly enhanced the concentrations of specific macronutrients, including P by 38%, K by 43%, and Ca by 6.5%. Notably, Ca was the only mineral consistently affected across most Se treatments (except the combined Mix treatment), showing a significant increase under both Se(VI) and Se(IV) applications, regardless of the presence of the biostimulant.

The stability of other analyzed mineral elements suggests that foliar Se biofortification does not substantially disrupt nutrient balance in alfalfa, indicating no antagonistic effects of Se on nutrient uptake. This aligns with the importance of studying the concentrations of essential elements in plants, as such analyses are pivotal for assessing both the efficiency of

the biofortification process and its impact on crop nutritive value while also monitoring potential ion imbalances that could reduce growth [50].

3.1.5. Selenium Speciation Analysis by XAS in Alfalfa Plants Grown Under Controlled Conditions

Figure 3 depicts the Se K-edge X-ray absorption near edge structure (XANES). The comparison of the different samples reveals the exchange in weight of XANES features characteristic of particular Se species, as revealed by the comparison with the references (top group on Panel A). In particular, the presence of Se organic species is contributing around 12.657 keV, while the presence of inorganic Se(IV) and Se(VI) species rises the spectral weight around 12.660 and 12.664 keV, respectively. The Se XANES in the alfalfa grown at laboratory scale presents both organic and inorganic spectra contributions.

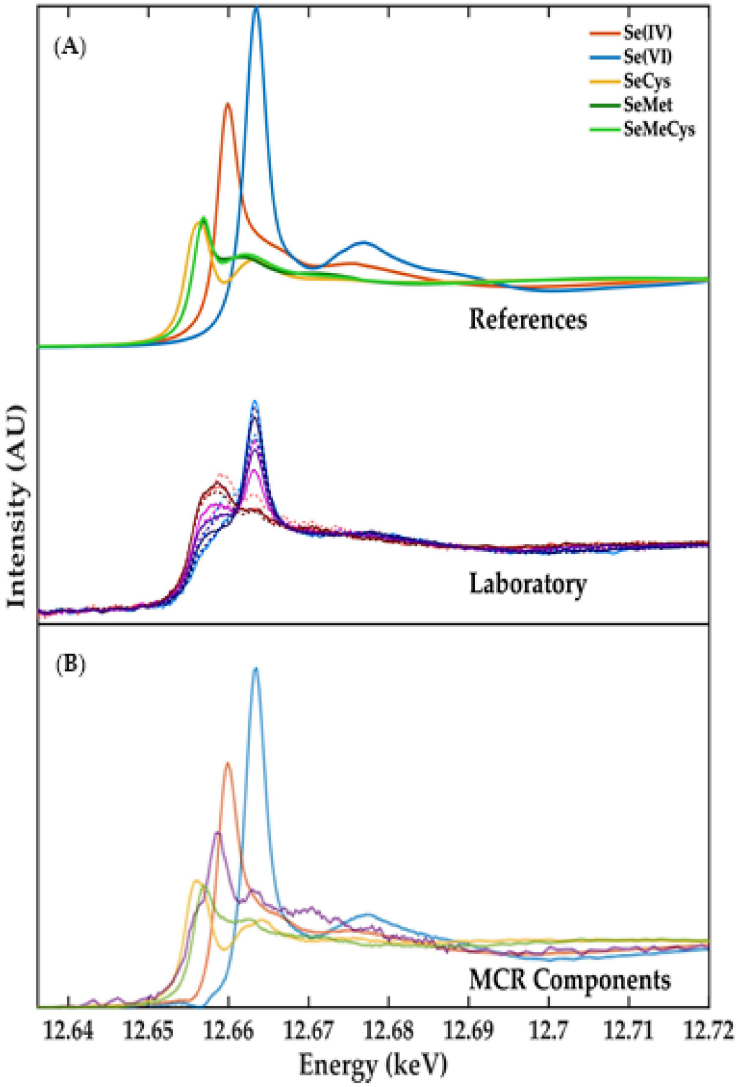

Figure 3. (**A**) Se K-edge XANES spectra collected over the aerial part of alfalfa plants grown under different selenium treatments in controlled conditions and compared with references (top row) and (**B**) with the obtained MCR components. Different selenium treatments are represented with different colors, where blue, red and violet corresponds to Se(VI), Se(IV) and Se(Mix); dark and light colors to 90 and 45 g ha^{-1} of dose; and continuous and dashed lines to with and without biostimulant, respectively.

To correctly deconvolute the sample spectra in their major components and to investigate the eventual presence of other contributions, multivariate curve resolution was applied [54]. The spectra of the five chemical components resolved by MCR are displayed

in Figure 3B. The white line energy positions of the one, two, three, four, and five MCR components correspond to 12.663, 12.659, 12.656, 12.657, and 12.656 keV, respectively. The comparison with the reference spectra allows to assign Components 1, 2, 3, and 5 to Se(VI), Se(IV), SeCys, and SeMet/SeMetCys species, respectively. Due to the very close similarity of the coordination environment of Se in SeMet and SeMetCys references (C-Se-C), it was not possible to distinguish between these two spectral contributions and they were described by a single MCR component. Interestingly, an additional component with a spectroscopic signature between organic-Se and Se(IV) species was also found, here labelled as Component 4.

The relative concentrations of the MCR components for the laboratory cultivations are depicted in Figure 4. The results indicate that Component 5 (SeMet/SeMeCys) is the predominant species, accounting for approximately 50% of the Se species present in the samples. This confirms the expected transformation of inorganic Se into organic forms. Components 3 (SeCys) and 4 (unknown Se species) are found in higher concentrations in samples treated with Se(IV). In contrast, the presence of inorganic Se species, particularly Se(VI), is maximized in samples subjected to Se(VI) treatment, while approximately 10% of Se(IV) is consistently detected across all samples.

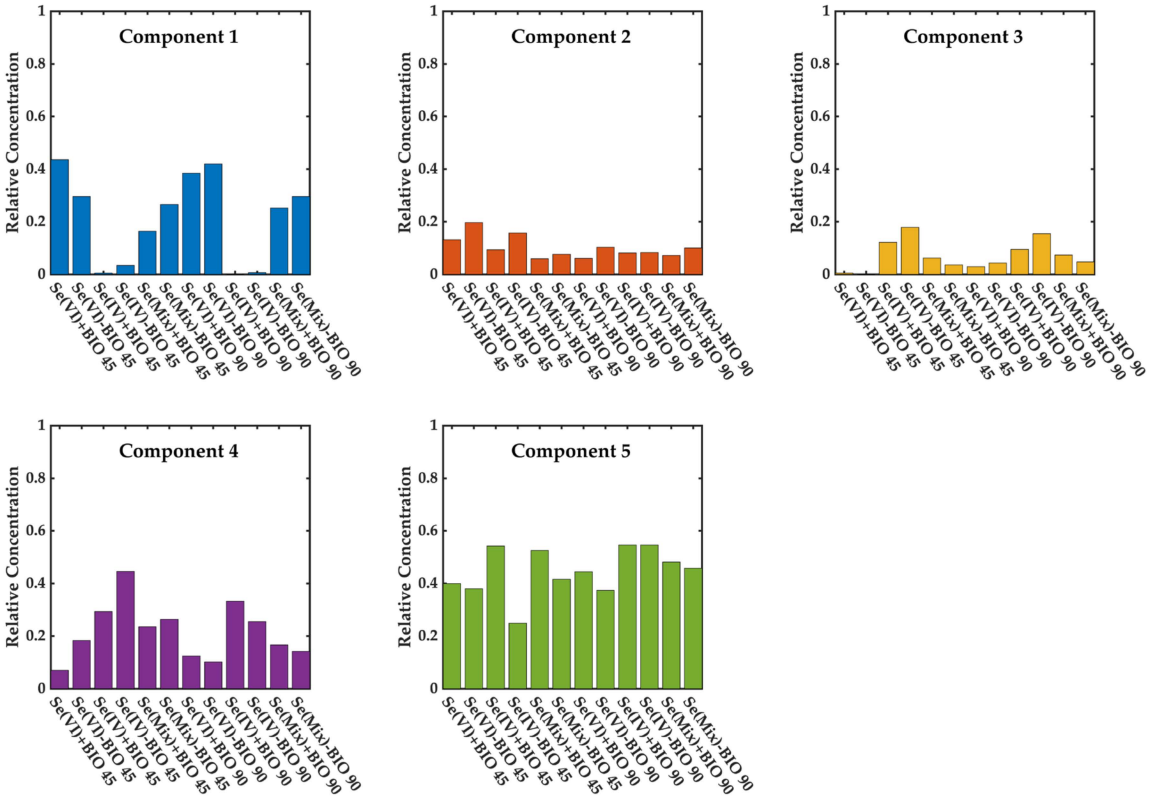

Figure 4. MCR component relative concentrations for the laboratory culture. Components 1, 2, 3, and 5 correspond to Se(VI), Se(IV), SeCys, and SeMet/SeMetCys, respectively. Component 4 represents unknown Se species.

In Figure 5, the relative MCR concentrations are presented as a function of Se content, distinguishing between Se(IV) (left), Se(Mix) (middle), and Se(VI) (right) applications.

The data indicate that Components 4 and 5 are present in greater amounts when Se(IV) is provided, while Component 1 increases and Components 3, 4, and 5 decrease with the amount of Se(VI) in the feeding. Component 4 seems to decrease by increasing the amount

of Se accumulated by the plants, suggesting that different mechanisms for Se conversion are activated depending on the Se accumulation level.

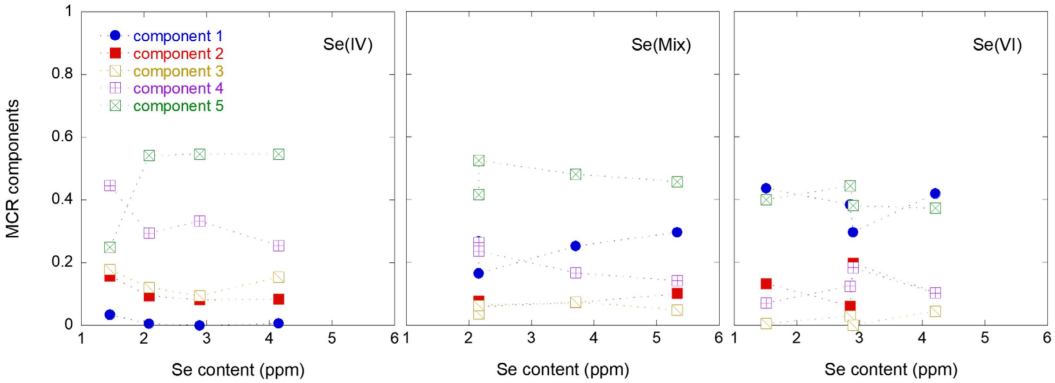

Figure 5. MCR concentrations as a function of selenium content, distinguishing between Se(IV) (left), Se(Mix) (middle), and Se(VI) (right) applications.

The figure highlights that total Se accumulation is enhanced by the combined application of Se(IV) and Se(VI). However, under Se(VI) treatment alone, plants appear less efficient in fully converting Se into organic forms compared to Se(IV) applications. This difference may be influenced by enzymatic processes regulating the reductive pathway, including the role of ATP sulfurylase and other key enzymes involved in Se assimilation. These enzymes facilitate the incorporation of Se into amino acids such as selenocysteine and selenomethionine, which could explain the observed differences in conversion efficiency between Se(IV) and Se(VI) treatments. Additionally, the kinetics of biotransformation differ between Se(IV) and Se(VI). Se(IV) is rapidly taken up by plant roots through phosphate transporters and is quickly assimilated into organic Se compounds within the roots. In contrast, Se(VI) is absorbed via sulfate transporters and translocated to the shoots before being reduced and incorporated into organic forms. This distinct uptake and translocation behavior contributes to the variations in biotransformation rates and efficiencies observed between Se(IV) and Se(VI) treatments.

Based on these findings, treatments with Se(IV) were selected for subsequent evaluation in open-field experiments with alfalfa plants, given their greater efficiency in promoting organic Se transformation.

The correlations between the composition of the Se species according to the MCR analysis, nutrient analysis, and agronomic parameters are illustrated in Figure 6 as a heat map for laboratory cultivation.

As shown in Figure 6, Component 3 (SeCys) correlates positively with Component 4 (unknown Se species) and negatively with Component 1 (Se(VI)). Additionally, Component 1 (Se(VI)) anticorrelates with *gs* and *E*. Component 2 (Se(IV)) shows a positive correlation with root fresh weight (*Root FW*), while Component 5 (SeMet/SeMeCys) does not display strong correlations with most variables except for K.

Regarding the elemental concentrations, Ni is positively correlated with Fe, Mn, and Se, while Zn correlates with Cu. Selenium also correlates with Mn and Ni. Photosynthetic parameters, including *E*, *gs*, and *Pn*, show strong positive correlations among themselves. Acid detergent fiber (*ADF*) correlates with neutral detergent fiber (*NDF*), *Pn*, and branch number (*Nr. Branches*). Phosphorus also correlates positively with Nr. Branches.

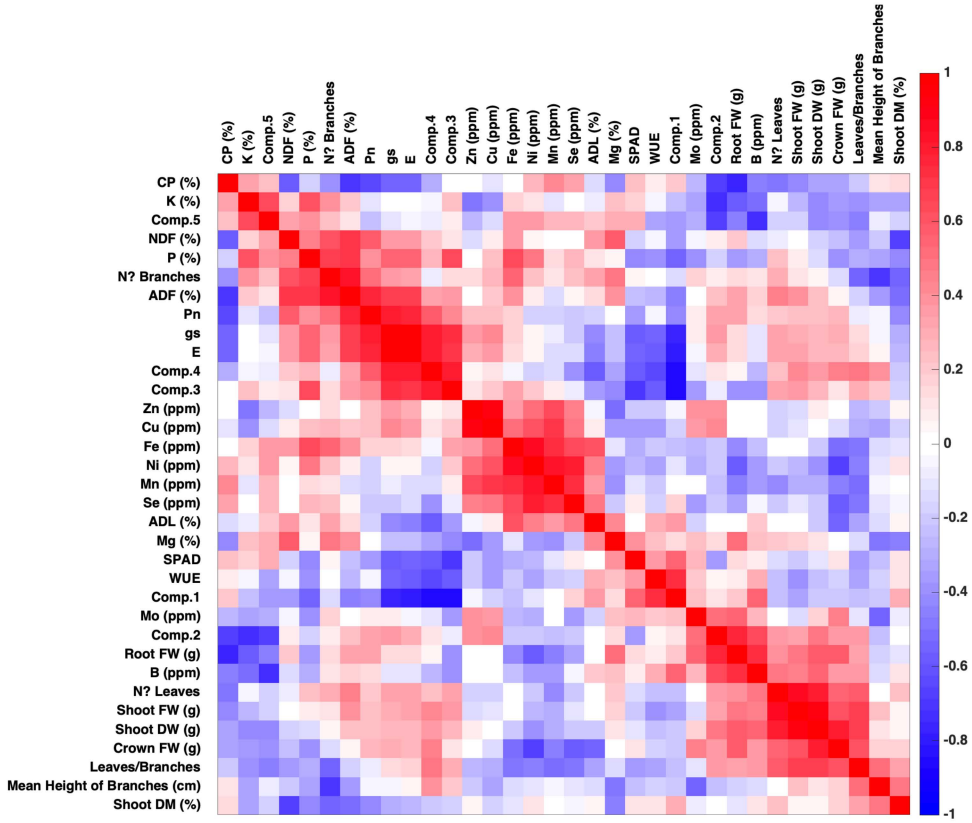

Figure 6. Correlation map between Se species concentrations and agronomic parameters.

Leaf number (*Nr. Leaves*) is positively correlated with shoot dry weight (*Shoot DW*) and shoot fresh weight (*Shoot FW*). Conversely, Root FW is negatively correlated with crude protein (*CP*), while Shoot DW positively correlates with crown fresh weight (*Crown FW*). Additionally, Root FW correlates with B.

3.2. Se-Biofortified Alfalfa at Open-Field

3.2.1. Effects of Selenium on Yield and Forage Quality

Table 1 presents the physicochemical composition data of alfalfa pasture from the trial conducted during the spring 2022 to summer 2023 period for the different treatments evaluated.

The results indicate no significant differences in alfalfa fresh matter production across the different treatments evaluated, nor in its chemical composition (DM, CP, NDF, ADF, ADL, EE, and ash). These findings are consistent with those obtained in controlled alfalfa growth trials. The nutritional parameters align with those reported by Berone et al. [46] for second-year alfalfa pastures at the same phenological stage in Argentina, which reported CP at 21% and NDF at 39.7%.

The application of the biostimulant did not affect the nutritional parameters, despite the atypical climatic conditions during the trial, which included an average temperature 4.3 °C higher and 50.5 mm less rainfall compared to the historical average for the region (source: siga.inta.gob.ar).

Table 1. Fresh weight production and percentages of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), ether extract (EE), and ash for the different treatments evaluated during the spring 2022 to summer 2023 period.

			Fresh Weight (g 0.25 m^{-2})	DM (%)	CP (%)	NDF (%)	ADF (%)	ADL (%)	EE (%)	Ash (%)
CONTROL		**(−) BIO**	376.8 ± 77.34 a	19.4 ± 0.98 a	23.6 ± 1.34 a	37.0 ± 3.84 a	26.2 ± 3.55 a	6.4 ± 0.68 a	2.5 ± 0.22 a	10.7 ± 0.38 a
		(+) BIO	362.9 ± 76.27 a	19.5 ± 0.85 a	22.4 ± 4.16 a	38.0 ± 6.14 a	26.4 ± 4.78 a	6.7 ± 0.97 a	2.6 ± 0.44 a	10.5 ± 0.39 a
Se(IV)	**45 g ha^{-1}**	**(-) BIO**	353.4 ± 92.08 a	19.0 ± 1.59 a	21.9 ± 2.74 a	39.6 ± 3.05 a	28.8 ± 3.12 a	7.5 ± 0.69 a	2.7 ± 0.46 a	10.4 ± 0.49 a
		(+) BIO	370.3 ± 68.05 a	19.4 ± 0.82 a	23.0 ± 2.66 a	36.5 ± 3.26 a	26.4 ± 2.55 a	6.8 ± 1.29 a	2.6 ± 0.28 a	10.5 ± 0.38 a
	90 g ha^{-1}	**(-) BIO**	355.8 ± 66.51 a	18.6 ± 2.66 a	22.4 ± 2.42 a	35.6 ± 4.42 a	25.6 ± 4.16 a	6.7 ± 0.98 a	2.7 ± 0.34 a	10.4 ± 0.62 a
		(+) BIO	361.7 ± 76.56 a	18.8 ± 1.28 a	21.0 ± 3.16 a	37.2 ± 4.47 a	27.1 ± 4.20 a	6.8 ± 1.24 a	2.7 ± 0.47 a	10.6 ± 0.33 a

Note. -BIO: without biostimulant; +BIO: with biostimulant (BIOFORGE® 600 mL ha^{-1}). Results are expressed as mean (n = 3) ± SE. Means not sharing any letter are significantly different based on the LSD test at $p < 0.05$.

3.2.2. Effect of Foliar Selenite Application on Selenium Content in Alfalfa Plants Grown Under Open-Field Conditions

Under open-field conditions, the foliar application of Se(IV) significantly increased the total Se content in alfalfa plants, both with and without a biostimulant (Figure 7). Compared to the controls, Se content increased by 11.4 and 7 times with 45 g ha^{-1} Se(IV) applied without and with a biostimulant, respectively, and by 19.3 and 10.1 times with 90 g ha^{-1} Se(IV) applied without and with a biostimulant, respectively. These results demonstrate a clear dose-dependent response, with higher Se accumulation observed at the 90 g ha^{-1} dose. Interestingly, Se concentrations were consistently lower in plants treated with the biostimulant, suggesting a modulatory effect on Se uptake. This effect may be attributed to physiological changes, such as altered leaf cuticle permeability or modified metabolic pathways involved in nutrient assimilation. Similar trends were observed in prior controlled environment experiments, where foliar Se applications significantly increased Se content in alfalfa (Figure 2), further corroborating these field-based findings. These results align with previous studies [28,29], which report a linear increase in Se content with higher application rates, though these increases were influenced by factors such as co-applied substances or environmental conditions. The observed reduction in Se accumulation with biostimulant treatments may reflect a trade-off between stress adaptation mechanisms and nutrient assimilation pathways, as reported in prior studies [55]. However, the precise mechanisms underlying this effect remain unclear. Several potential explanations can be considered, including alterations in leaf cuticle permeability, interference with Se transporter expression, and modulation of plant hormone signaling.

Biostimulants are known to modify leaf cuticle permeability, influencing the uptake of various substances, including nutrients and pollutants [56]. Although cuticle permeability was not directly assessed in this study, it is plausible that BIOFORGE® altered cuticle properties, thereby reducing Se uptake. Future studies could address this hypothesis by analyzing cuticle thickness, composition, and permeability in treated and untreated plants.

Another possible mechanism involves the regulation of Se transporter expression. Plants possess specific transporters responsible for Se uptake and translocation [56], and biostimulants could interfere with their expression or activity, ultimately limiting Se accumulation in plant tissues.

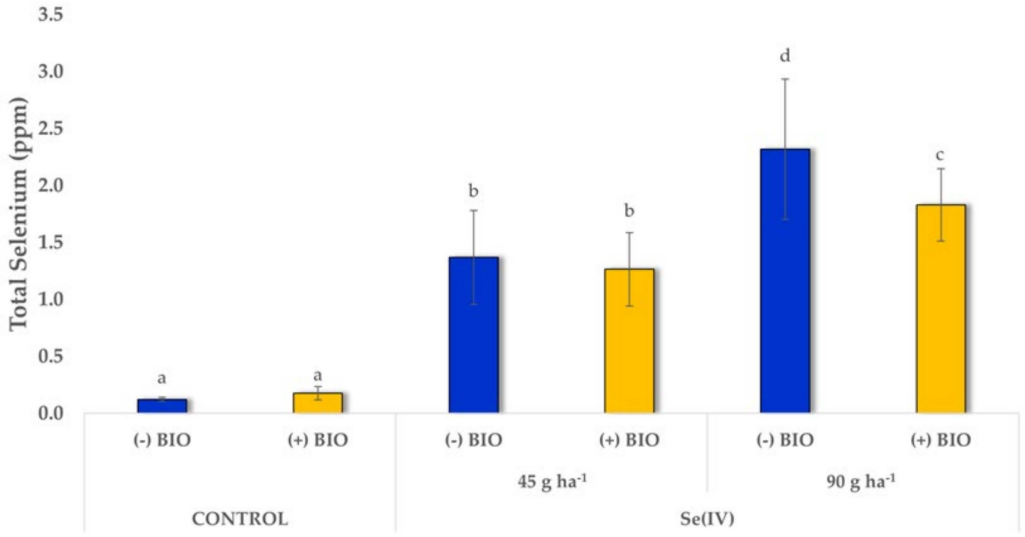

Figure 7. Total selenium concentration in the aerial parts of alfalfa plants grown under different Se treatments in open-field conditions. -BIO: without biostimulant; +BIO: with biostimulant (BIOFORGE® 600 mL ha^{-1}). Results are expressed as the mean (n = 3) ± SE. Means not sharing any letter are significantly different according to the LSD test at $p < 0.05$.

Plant hormones may also play a role in the observed reduction of Se uptake. For example, abscisic acid (ABA) regulates stomatal closure, potentially affecting the absorption of various substances, including Se [57]. It is possible that BIOFORGE® influences hormone levels or signaling pathways, indirectly modulating Se uptake. Future investigations could explore this aspect by quantifying hormone levels in plant tissues or using hormone-specific inhibitors or mutants.

3.2.3. Effects of Selenium on Mineral Elements in Alfalfa Plants Grown Under Open-Field Conditions

The results indicate that the foliar application of Se(IV) had minimal impact on the concentrations of micronutrients such as B, Mn, Fe, Ni, Zn, Cu, and Mo, as no significant differences were observed across treatments compared to the control, regardless of the presence of a biostimulant (Table S6). This stability suggests that foliar application of Se does not interfere with the uptake of these micronutrients in alfalfa plants under open-field conditions.

In terms of macronutrients (Table S6), Mg concentrations exhibited slight variation across treatments, with a significant increase of approximately 8% in the presence of the biostimulant compared to the control without the biostimulant. Phosphorus levels remained stable across all treatments, averaging between 0.33% and 0.35%. Similarly, no significant differences in Ca concentrations were detected across treatments. Sulphur concentrations also remained consistent, indicating no antagonistic effects between Se and S assimilation. In contrast, K content was significantly enhanced by approximately 8.5% with the application of 45 g ha^{-1} Se(IV), irrespective of biostimulant application.

Recent studies have shown that foliar Se applications can influence the nutrient composition of plants, depending on both Se dosage and plant species [58,59]. For instance, foliar Se treatments in soybeans have been reported to increase the concentrations of K, P, and S in certain genotypes while enhancing Mn and Fe levels in others [59]. These findings suggest that foliar Se applications not only increase Se content but may also modulate the accumulation of essential nutrients such as K, which are critical for plant growth and stress resilience. However, these effects appear to be genotype-specific, highlighting the potential variability in nutrient modulation based on plant variety [59].

3.2.4. Selenium Speciation Analysis by XAS in Alfalfa Plants Grown Under Open-Field Conditions

Figure 8 shows the Se K-edge XANES spectra collected from Se-biofortified alfalfa grown under open-field conditions. The XAS spectra across the different samples are similar and are predominantly characteristic of organic Se species.

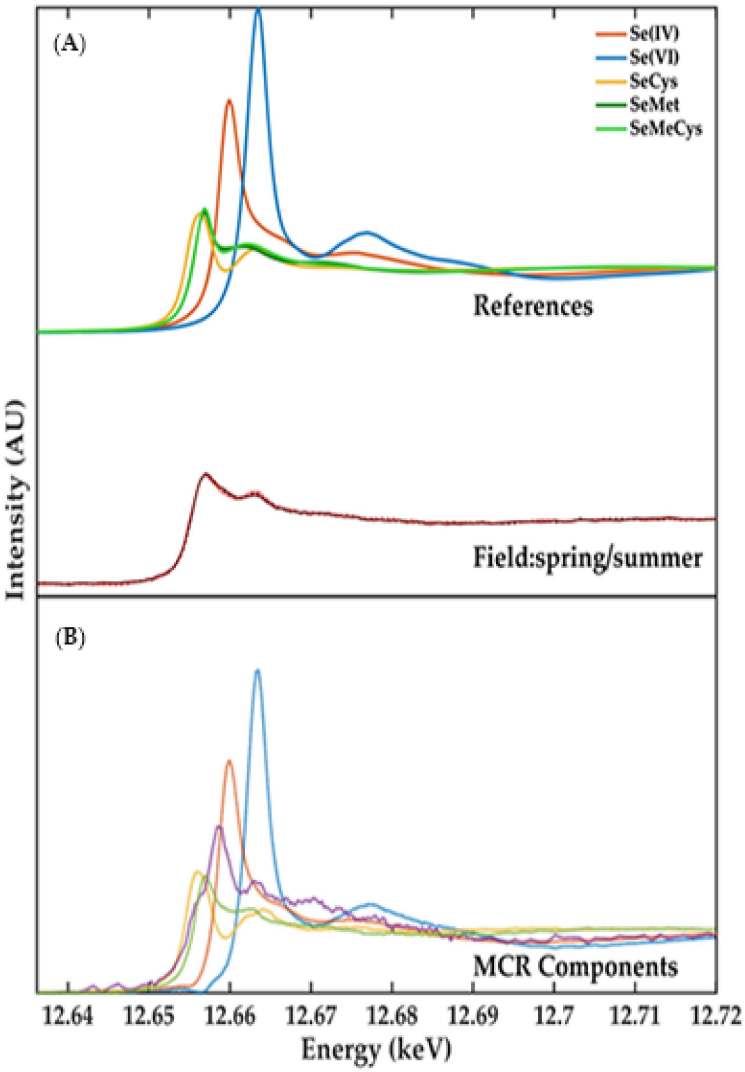

Figure 8. (**A**) Se K-edge XANES spectra collected from alfalfa plants grown under different selenium treatments in open-field conditions, compared with references and (**B**) the MCR components obtained. The different treatments are represented with distinct colors: dark and light shades correspond to doses of 90 and 45 g ha^{-1}, respectively; continuous and dashed lines indicate treatments with and without the biostimulant, respectively.

The relative concentrations of the MCR components for the open-field cultivations are presented in Figure 9. Similar to the laboratory trials, Component 5 (SeMet/SeMeCys) is the predominant species in alfalfa grown under open-field conditions across all treatments. Interestingly, the proportion of Component 5 increases from approximately 50% in laboratory-grown plants to 60% in open-field conditions. Unlike laboratory-grown alfalfa, open-field plants show negligible amounts of inorganic-Se species, confirming a more complete transformation of inorganic-Se into organic-Se species.

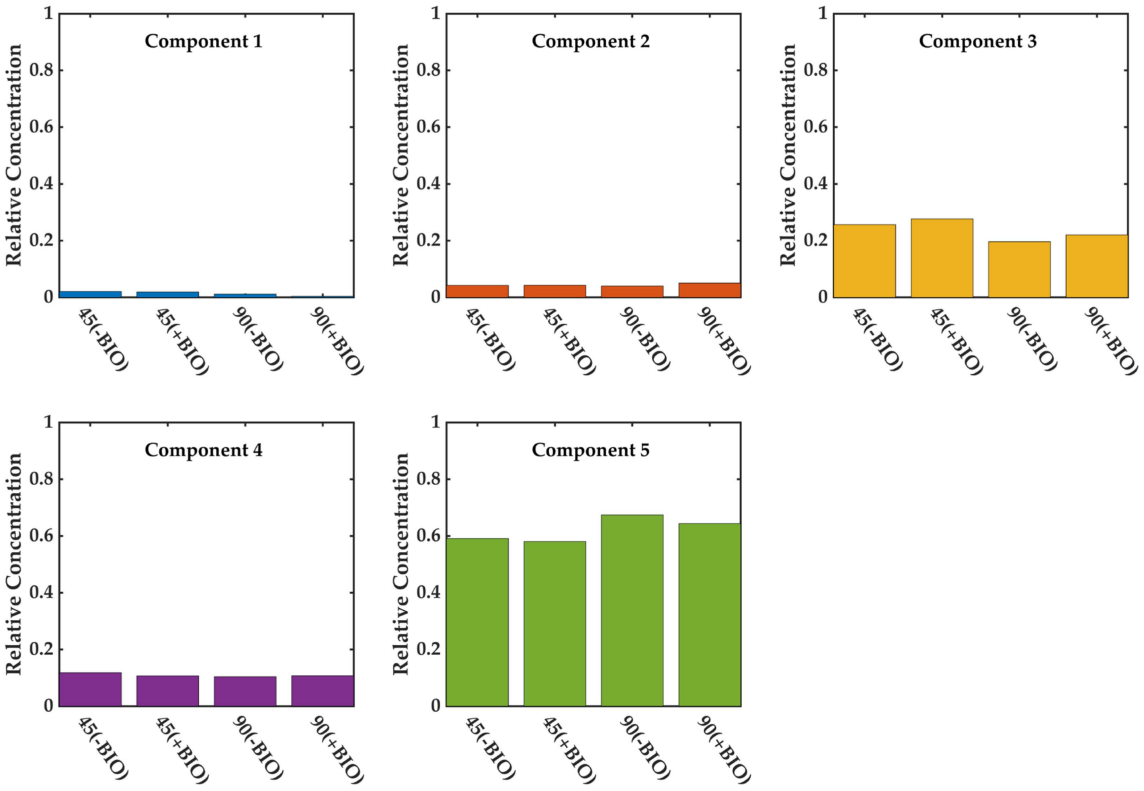

Figure 9. MCR component relative concentrations for open-field cultures. Components 1, 2, 3, and 5 correspond to Se(VI), Se(IV), SeCys, and SeMet/SeMeCys, respectively. Component 4 represents unknown Se species.

Component 3 (SeCys) accounts for 20–25% of the Se species detected in open-field plants, slightly higher than in laboratory conditions treated with Se(IV). Additionally, Component 4 (unknown Se species) decreases from 30–40% in laboratory plants to around 10% in open-field conditions, suggesting a possible conversion between these unknown species and the organic Se species, indicating a more efficient transformation in the latter.

The correlations among the composition of the Se species according to the MCR analysis, elemental analysis, and agronomic parameters for open-field cultivations are illustrated as a heat map in Figure 10. Notably, Se(IV) treatment exhibits stronger correlations in this context. Unlike the laboratory results, Component 3 (SeCys) shows a positive correlation with K and a negative correlation with Component 5, Se, and Ca. Additionally, Component 1 (Se(VI)) correlates positively with K and negatively with Mo, Se, Ca, Na, Zn, and S. Component 2 (Se(IV)) correlates positively with Fe, ash, S, Cu, and P, while showing a negative correlation with CP.

Furthermore, Component 5 (SeMet/SeMeCys) exhibits a negative correlation with Component 3, K, Se, and Ca, contrasting with observations from laboratory trials. Component 4 (unknown Se species) correlates positively with K, ADL, NDF, ADF, and Mg, while negatively correlating with Ca, Na, B, and Zn.

In terms of elemental concentrations, Ni correlates positively with Mg and negatively with green matter (GM), while GM shows a negative correlation with Mg. K is positively correlated with ADL, NDF, and ADF, but negatively correlated with Se, Ca, Na, and B, aligning with Components 1, 3, and 4, but contrasting with Component 5. Conversely, Ca negatively correlates with ADL and NDF, while showing positive correlations with Se, Na, B, and Zn, consistent with Component 5. Mn, Mo, Fe, and EE correlate positively with each other and negatively with CP. Finally, Se concentration positively correlates with Component 5 and Ca, but negatively correlates with Components 1 and 3, as well as K.

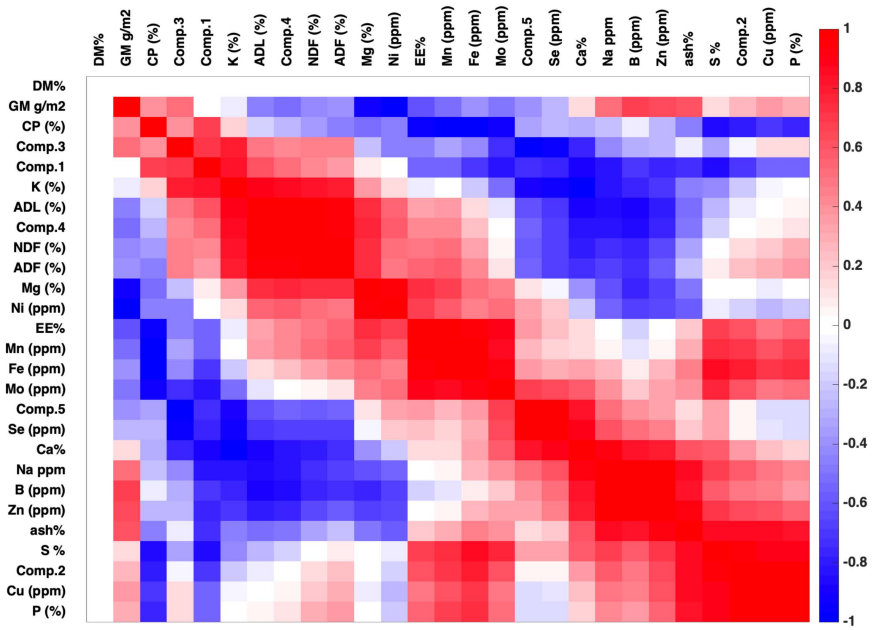

Figure 10. Correlation map between the Se species concentrations and the agronomic parameters for open-field cultivations.

4. Conclusions

This study demonstrated that Se biofortification in alfalfa through the foliar application of Se salts (selenate, selenite, or a combination), with and without a biostimulant, is an effective approach to enhance Se accumulation and transformation into bioavailable organic forms. Laboratory and open-field trials confirmed that higher application doses (90 g ha^{-1}) of Se significantly increased Se concentration, with selenite (Se(IV)) showing notable efficacy. Although the co-application of a biostimulant slightly reduced Se accumulation, it did not negate the biofortification benefits. Physiologically, Se application—particularly with selenite—positively influenced parameters such as net photosynthesis rate (Pn), stomatal conductance (gs), and transpiration rate (TRN), without adversely affecting chlorophyll content, indicating non-toxic levels. Forage quality, assessed through crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), remained unaffected, ensuring that Se application does not compromise feed value. Mineral composition analysis highlighted increases in manganese (Mn), iron (Fe), and nickel (Ni), with minimal impact on other micronutrients. Potassium (K) levels increased at lower application rates under field conditions. X-ray absorption spectroscopy (XANES) revealed a significant conversion of inorganic Se species (Se(VI) and Se(IV)) into organic forms such as selenomethionine (SeMet) and methyl–selenocysteine (MeSeCys), with field conditions showing a more pronounced transformation compared to laboratory settings. These findings underline the potential of Se-enriched alfalfa as a sustainable source of bioavailable organic selenium for animal and potentially human nutrition.

In conclusion, the foliar application of Se, particularly as selenite (Se(IV)), represents a practical and effective strategy for Se biofortification in alfalfa without compromising forage quality or mineral composition. The study highlights the importance of tailoring Se biofortification strategies to environmental conditions and emphasizes the role of this approach in promoting sustainable agricultural practices while enhancing the nutritional value of alfalfa-derived products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15030580/s1, Table S1: Chemical properties of soil (0-0.20-m

depth) in the experimental site (31.35 S, 61.49 W) [60,61]; Table S2: Effect of foliar selenium application on leaf unit transpiration rate (TRN), stomatal conductance (gs) and leaf chlorophyll index (SPAD) in alfalfa plants under different Se treatments in controlled conditions; Table S3: Effect of foliar selenium application on yield parameters in alfalfa plants grown under different Se treatments in controlled conditions; Table S4: Effect of foliar selenium application on forage quality parameters, including percentages of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) in alfalfa plants grown under different Se treatments in controlled conditions; Table S5: Concentrations of micronutrient (B, Mn, Fe, Ni, Zn, Mo) and macronutrient (Mg, P, K, Ca, S) in the aerial parts of alfalfa plants grown under different Se treatments in controlled conditions; Table S6: Concentrations of micronutrient (B, Mn, Fe, Ni, Zn, Mo) and macronutrient (Mg, P, K, Ca, S) in the aerial parts of alfalfa plants grown under different Se treatments in open-field conditions.

Author Contributions: Conceptualization, M.-J.S.-M., M.G., R.B., M.V. and F.F.M.; Data curation, M.G., L.S., C.M., F.M. and R.B.; Formal analysis, L.S., C.M., F.M. and R.B.; Funding acquisition, M.-J.S.-M., R.B. and M.V.; Investigation, M.G., G.C., M.M.S., M.V.-P., J.M.P., M.G.G. and F.F.M.; Methodology, M.G. and F.F.M.; Project administration, M.-J.S.-M. and R.B.; Resources, M.G., L.S., F.M., M.L., M.V. and J.M.P.; Software, L.S., C.M. and F.M.; Supervision, M.-J.S.-M., M.G. and F.F.M.; Validation, M.-J.S.-M., M.G., M.L. and F.F.M.; Visualization, M.G., L.S. and F.F.M.; Writing—original draft, M.-J.S.-M., M.G., L.S., C.M., F.M., G.C., M.M.S., M.V.-P., J.M.P. and F.F.M.; Writing—review and editing, M.-J.S.-M., M.G., R.B., M.L., M.V., G.C., M.G.G. and F.F.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declare that the present work was conducted in the absence of any commercial or financial relationship that could be considered a potential conflict of interest. All authors read and approved the final manuscript.

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