**Title and authorship**

**Title:** Effects of asparagus decline on nutrients and phenolic compounds, spear quality, and allelopathy

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**Abstract**

Asparagus (*Asparagus officinalis*) decline is an important issue that decreases both crop production and quality causing economic losses. The main bioactive compounds in asparagus are phenolics. Several studies have related these compounds with the decline and also with the replanting issues in which when asparagus plants are replaced by new plants, their productivity is low. The asparagus physiological state and the spear quality have not been studied yet in field plots showing decline symptoms. Therefore, the aim of this study was to study the effect of asparagus decline on leaf nutrients accumulation and on the yield and quality of commercial spears. Another aim was to analyze the possible allelopathic effect on another species. For this, mineral nutrients, phenolic compounds and quality indicators were analyzed in plants and spears from two asparagus plots (with and without decline symptoms) and germination tests were conducted using lettuce seeds. Decline plants did not show great changes in macronutrient concentration but they showed low B and high Fe concentrations. Leaves and rhizomes from the plot with decline presented higher phenolic compounds, highlighting caffeic acid in rhizomes. However, spears did not present higher phenolic compounds. Likewise, decline decreased the market spear quality and yield but slightly increased some quality indicators such as chlorophylls, carotenoids, ascorbate, and antioxidant capacity. On the other hand, extracts from the decline plot reduced lettuce germination and epicotyl growth suggesting an allelopathic effect. The obtained results are useful to a better understanding of the decline issue and provided valuable information for producers to manage and control its effects on asparagus quality.

**Keywords:** Allelopathy, Antioxidants, Asparagus (*Asparagus officinalis*), Decline, Phenolic compounds, Quality

**1. Introduction**

Asparagus (*Asparagus officinalis*) production has great socio-economic importance. Thus, in 2016, it reached 16.4 million tons worldwide. Spain production ranks in 6th position with 59,881 tons and is also the 2nd most important producer in the EU (FAO, 2018). Granada region (Andalusia, Spain) presents the 80% of the national production (Consejería de Agricultura, Pesca y Desarrollo Rural, 2015).

Asparagus is well known for its organoleptic characteristics and its wealth of nutrients and bioactive compounds (Fuentes-Alventosa et al., 2008; Sanae & Yasuo, 2013). Thus, it was reported as the plant with the highest content of antioxidant compounds among the vegetables commonly consumed in the United States and Europe. The main bioactive compounds in asparagus are phenolics, including phenolic acids and flavonoids, besides important nutrients such as ascorbic acid (Vitamin C) and glutathione (Rodríguez et al., 2013). The nutrients and phytochemicals in asparagus may act as health promoters capturing free radicals, preventing cell oxidation, and also preventing heart disease and certain cancers (Lu et al., 2010; Sanae & Yasuo, 2013).

On the other hand, from an agronomical point of view, phenolic compounds may be involved in phytotoxicity or autotoxicity phenomena in crops (Kato-Noguchi et al., 2017; 2018). Thus, autotoxicity is produced when an annual crop is replanted in the same field or in cultures of the perennial crops for several years. Examples of these multiyear crops are alfalfa (*Medicago sativa L.*), asparagus (*Asparagus officinalis L.*), and sugar cane (*Saccharum officinarum*) (Webber et al., 2018). Regarding asparagus, autotoxicity is attributed to the release of allelochemical compounds, mainly coumaric, caffeic, and ferulic acids (Elmer & Pignatello, 2011; Kato-Noguchi et al., 2018). Autotoxicity, also called "asparagus decline", is one of the main issues that affect this crop and it causes the decrease of both crop production and quality (Kato-Noguchi et al., 2017; Elmer, 2018). At the same time, the "replanting issue" was observed when asparagus plants were replaced with new plants, and both the crop productivity and quality remain low. Both problems may be caused by biotic and abiotic factors, being the crown rot disease caused by *Fusarium spp.* the main biotic factor, whereas allelopathy can be the major abiotic factor (Kato-Noguchi et al., 2017, 2018).

Likewise, Miller et al. (1991) investigated which compounds in asparagus roots could inhibit asparagus seeds germination and identified caffeic acid as a germination inhibitor. They also found that caffeic acid acted synergistically with *Fusarium oxysporum f. sp. Asparagi*.On the other hand, Kato-Noguchi et al. (2017; 2018) observed that methanol extracts from asparagus rhizomes inhibited the growth of roots and seedlings of asparagus in a dose-dependent manner. Thus, the previous results suggest that the extracts exert autotoxicity and contain some growth-inhibiting substances, released by asparagus, that inhibit its own growth. However, the plant's physiological state and the spear quality have not been studied yet in field plots that show decline symptoms. Therefore, the aims of this study were to study the effect of asparagus decline on the accumulation of macro and micronutrients in leaf and rhizomes and on the yield and quality of commercial spears. Another aim was to analyze the possible allelopathic effect of asparagus extracts on another species using a germination test to analyze the potential effect of allelopathy on other possible cultures in the same field. The hypothesis to test in this study was that there will be differences in nutritional status, asparagus quality, phenolics compounds between asparagus from a plot with decline and a plot without decline. An additional hypothesis is that extracts from the plot with decline will have an allelopathic effect on lettuce seeds germination.

**2. Materials and methods**

*2.1. Plant material*

A classification of 11 plots located in Granada, Spain, was carried out based on their decline symptoms, their variety and the years of cultivation. The samples selected for this study were from two different plots (one with decline symptoms and another without decline symptoms). Both plots are located in the municipality of Loja. The asparagus variety was Large F1, with more than 5 years of production and from San Isidro cooperative. Thus, in both plots were carried out the same agricultural practices. Asparagus organs: leaves, rhizomes, and spears were used as plant material.

*2.2. Mineral Nutrients*

The leaves and rhizomes samples were mineralized by wet digestion according to Wolf (1982). For this, 0.2 g of ground freeze-dried plant material was digested with 98% H2SO4 and 30% H2O2 at 300 °C. The phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) were analyzed by atomic absorption spectrometry. For organic nitrogen (N) determination, 20 ml of distilled water were added to the digests in order to test the total reduced nitrogen (N) concentration by colorimetry based on the Berthelot reaction, as described by Krom (1980).

*2.3. Phenolic compounds by HPLC-DAD-ESI-MSn analysis in leaves, rhizomes, and spear*

The freeze-dried samples were extracted with 70% methanol in an ultrasound bath for 1 h (shake vortex before, at 30 min, and after sonication). Then they were kept at 4 °C for 12 h and they were again sonicated for 1 h. Finally, they were centrifuged at 850 *g* for 15 min. The supernatant was collected and filtered through 0.22 μm PVDF, 13 mmØ. The HPLC-DAD-ESI/MSn analyses were carried out in an Agilent HPLC 1200 (Agilent Technologies, Waldbronn, Germany) coupled to a mass detector in series. The HPLC system consisted of a binary capillary pump (model G1376A), an autosampler (model G1377A), a degasser (model G1379B), a sample cooler (model G1330B), and a photodiode array detector (model G1315D). The mass detector was a Bruker ion trap spectrometer (UltraHCT model; Bruker, Bremen, Germany), equipped with an electrospray ionization interface (ESI). The ionization conditions were adjusted to 350°C and 4 kV for the capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L min-1, respectively. The full-scan mass covered the range from m/z 100 up to m/z 1200. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization. The MSn was carried out in the automatic mode on the most abundant fragment ion in the MS (n-1). For the RP-HPLC-DAD analysis, a C18 5u Kinetex Phenomenex column (250x4.6 mm) was used in a gradient of (A) Water + Formic 1% and (B) ACN 100%, to separate the compounds of interest in the samples. Compounds were quantified by HPLC-DAD using external standard curves chlorogenic acid and quercetin (Sigma Aldrich, Barcelona).

*2.4. Weight, diameter, and length of asparagus spears*

The spears were weighed on a pressure scale, then the length was measured in centimeters and finally, the diameter of each spear was measured using a Vernier caliper.

*2.5. Determination of photosynthetic pigments concentration in spear*

The photosynthetic pigments concentration was analyzed following the Wellburn et al. (1994)method with small modifications. 0.1 g of frozen asparagus spear was macerated in 1 mL of methanol and subsequently was centrifuged 5 min at 2200 *g*. The absorbance was measured at 3 different wavelengths: 653 nm, 666 nm, and 470 nm. The calculation was made in the following way:

Chlorophyll a = 15.65 X A666nm - 7.34 X A653nm

Chlorophyll b = 27.05 X A653nm - 11.21 X A666nm

Carotenoids = (1000 X A470nm - 2.86 X Cla - 129.2 X Clb) / 221

*2.6. Antioxidant capacity tests in asparagus spears*

For 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and reducing power tests, the asparagus spear was homogenized in 80% methanol and centrifuged at 3,000 *g* for 10 min. The DPPH free radical scavenging effect test was performed according to Hsu et al. (2003) with some modifications. An aliquot of the plant extract was mixed with 0.1 M DPPH and kept for 60 min in the dark and cold. The absorbance of the reaction mixture was then read at 517 nm. The effect of free radical elimination was calculated as follows: ROS removal effect (%) = [1- (A517, sample / A517, blank)] × 100.

The reducing power was measured following Hsu et al.(2003). The extract was mixed with phosphate buffer (0.2 M, pH 6.6) and K3Fe(CN)6 (1% v/w) and kept for 20 min at 50 °C. The sample was cooled immediately and then 10% Cl3CCOOH was added. After centrifugation at 3000 *g* for 10 min, the supernatant was mixed with distilled water and FeCl3 (0.1%). After 10 min the absorbance was recorded at 700 nm.

For Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) tests, the asparagus spear was homogenized in methanol and centrifuged at 12,800 *g* for 2 min. FRAP assay was performed using 1 mM, 2,4,6-tripyridyl-2-triazine and 20 mM FeCl3 in 0.25 M CH3COONa, pH 3.6. The absorbance was then measured at 593 nm (Benzie & Strain, 1996).

TEAC assay was conducted according to Re et al. (1999) and Cai et al. (2004) methods with modifications. Radical cation ABTS+ [2,2-azinobis-(3-acid-ethylbenzthiazoline-6-sulfonic)] was produced using 7 mM ABTS and 2.45 mM potassium persulfate incubate in the dark and at room temperature for 16 h. Then, the ABTS+ solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. The supernatant and the diluted ABTS+ solution were mixed, kept at room temperature for 6 minutes and the absorbance was measured at 734 nm.

*2.7. Reduced ascorbate (AsA) determination in asparagus spear*

The extraction and quantification of reduced AsA followed the Law et al. (1992) method. 0.5 g of the spear were homogenized with 5 ml of 5% metaphosphoric acid (w/v) and centrifuged at 4 °C for 15 min. Then, 0.2 ml of supernatant was mixed with 0.5 ml of 150 mM sodium phosphate buffer (pH 7.5) and 0.1 ml of 10 mM dithiothreitol (DTT). The mixture incubated at room temperature in the dark for 10 min. Then, 0.1 ml of N-ethylmaleimide 0.5% (w / v) was added along with 0.4 ml of orthophosphoric acid at 44% (v/v), 0.4 ml of 2,20-bipyridyl at 4% (w / v) in 70% ethanol and 0.2 ml of 3% FeCl3 (w/v). The resulting reaction mixture was incubated at 40 °C in the dark for 40 min. Finally, the absorbance was measured at 525 nm. The results were used to quantify the total AsA concentration, whereas the reduced AsA was quantified in the same way as the previous procedure, replacing 0.1 ml of DTT with 0.1 ml of distilled H2O.

*2.8. Phenolics extraction and phytotoxicity test*

The methodology of Kato-Noguchi et al. (2017) was used with some modifications. Rhizomes and asparagus leaves were cut into small pieces (the equivalent of 100 g of dry weight) and extracted with 500 ml of 70% (v/v) aqueous methanol for two days. After filtration using filter paper (No. 2, Toyo, Tokyo, Japan), the residue was put back into extraction with 500 ml of methanol for two days and filtered. Finally, both filtrates were mixed. The extracts obtained from 10, 30 and 50 mg of the vegetable tissue were subjected to 40°C for evaporation, then the residue was dissolved in 0.3 ml of methanol and added to a filter paper in a Petri dish of 3 cm. The methanol was evaporated in an extractor hood and 1 ml of 0.05% (v/v) Tween20 was added. Once this process finished, lettuce seeds (*Lactuca sativa*) were placed in the Petri dishes and incubated at 25°C with an 18 h photoperiod for 170 h. As a control treatment, methanol (0.3 ml) was added to the filter paper in the Petri dish and evaporated as described above. Germination was measured every 6 hours after the first seed sprouting, during 60 h, then it was measured every 12 h. The roots and the epicotyls lengths were measured after 170 h., we used the methodology proposed by FAO (1991) to obtain the germination percentage:

Germination percentage = ΣSG / n x 100

SG: Germinated seeds per plate

n: Total number of seeds sown

*2.9. Statistical analysis*

All analyses were repeated in triplicate and the results were statistically evaluated using a simple ANOVA variance analysis with a 95% confidence interval. The differences between the means of the treatments were compared using the Fisher's least significant differences (LSD) test at a 95% probability level. Statgraphics Centurion XVI software was used to perform the analyses. Significance levels were expressed as: \* P <0.05; \*\* P <0.01; \*\*\* P <0.001; NS not significant.

**3. Results and discussion**

*3.1. Nutrients in asparagus leaves and rhizomes*

Plant nutrients are chemical elements that are essential to complete the life cycle (Marschner 2011). An adequate nutritional supply could be important to mitigate asparagus decline. Thus, a balance of mineral nutrients is crucial to growing a healthy root system to withstand stress factors (Elmer, 2018). Our foliar macronutrient results showed that the P concentration was higher in leaves from the plot with decline than in plants from the plot without decline. However, this leaves showed lower Ca concentration. The other macronutrients showed no significant differences (Table 1).In rhizomes, the N content did not show significant differences between both plots. On the other hand, in the plot without decline the P, K, and Ca concentrations presented a significant increase in relation to the plot with decline (Table 1), while Mg and S were significantly lower in the plot without decline.Reuter and Robinson (1997) presented reference values ​​for asparagus cultivation (Table 1). When we compared our results of asparagus leaves with these values, N, P, K, Mg and Ca were within the optimal ranges in plants from both plots. Regarding S, no reference ranges were found in the asparagus crop and the results did not show significant differences between the plots. Therefore, results suggested that the macronutrient concentration in leaves and roots did not show a detrimental decline effect.

**Table 1. Macronutrients concentration in asparagus leaves and rhizomes and reference values in the cultivation of asparagus**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Macronutrients (mg g-1 D.W.)** | | | | | | |
| **Leaves** | **N** | **P** | **K** | **Ca** | **Mg** | **S** |
| Without decline | 25.61 ± 1.64 | 3.84 ± 0.07 | 16.74 ± 1.22 | 8.28 ± 0.44 | 2.07 ± 0.18 | 3.44 ± 0.38 |
| Decline | 27.87 ± 1.05 | 4.37 ± 0.06 | 14.12 ± 0.24 | 4.49 ± 0.15 | 1.66 ± 0.02 | 3.15 ± 0.06 |
| *p-value* | NS | \*\* | NS | \*\* | NS | NS |
| LSD | 4.11 | 0.25 | 3.44 | 1.29 | 0.51 | 1.06 |
| **Rhizomes** |  |  |  |  |  |  |
| Without decline | 10.27 ± 0.56 | 1.46 ± 0.11 | 12.14 ± 0.72 | 4.49 ± 0.15 | 0.70 ± 0.05 | 2.13 ± 0.11 |
| Decline | 9.11 ± 0.28 | 0.82 ± 0.02 | 4.94 ± 0.01 | 3.17 ± 0.39 | 1.17 ± 0.07 | 3.04 ± 0.14 |
| *p-value* | NS | \*\* | \*\*\* | \* | \*\* | \*\* |
| LSD | 1.32 | 0.30 | 1.99 | 1.15 | 0.23 | 0.50 |
| **Asparagus macronutrients reference concentrations (mg g-1 D.W.)** | | | | | | |
|  | 22-29 | 3-5 | 15-24 | 4-8 | 1.5-3.0 | - |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control. Reference values according to Reuter and Robinson (1997)

Plants present low micronutrient concentrations compared to macronutrient, however, they are crucial for plants to complete their life cycle (Marschner 2011). Table 2 results show that leaves from the plot with decline presented lower B and Mn but higher Fe and Zn concentrations. Previous studies associated Mn concentration with asparagus health (Elmer, 2018). Thereby, the lower Mn concentration in asparagus leaves might be a symptom of decline. On the other hand, rhizomes from plants with decline registered higher B, Fe, and Mn values. However, Zn and Cu did not show significant differences between plots (Table 2). In comparison with reference values ​​provided by Reuter and Robinson (1997),it was found that concentrations Mn, Zn, and Cu were within the optimum ranges in both plots. Nevertheless, B presented sub-optimal levels, probably causing B deficiency in the plant. On the other hand, Fe presented an optimum concentration in the plot without decline, whereas in the decline plot its concentration was 25% greater than the upper limit of the reference values (Table 2). In short, decline plants showed an alteration in their micronutrient profiles. Thus, plants presented potentially toxic Fe values in the plot with decline, whereas they showed a B deficiency in both plots, being more severe in the plot with decline.

**Table 2. Micronutrients concentration in asparagus leaves and rhizomes and reference values in the cultivation of asparagus**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Micronutrients (μg g-1 D.W.)** | | | | | |
| **Leaves** | **B** | **Fe** | **Mn** | **Zn** | **Cu** |
| Without decline | 32.03 ± 3.02 | 107.59 ± 3.48 | 77.69 ± 5.40 | 23.24 ± 0.99 | 6.96 ± 0.22 |
| Decline | 19.35 ± 0.57 | 313.33 ± 65.40 | 31.76 ± 1.30 | 30.73 ± 1.03 | 6.75 ± 0.29 |
| *p-value* | **\*** | **\*** | **\*\*** | **\*\*** | NS |
| LSD | 8.52 | 181.84 | 15.43 | 3.98 | 1.01 |
| **Rhizomes** |  |  |  |  |  |
| Without decline | 6.90 ± 0.36 | 313.33 ± 65.40 | 22.38 ± 1.74 | 16.24 ± 1.07 | 6.67 ± 0.41 |
| Decline | 12.05 ± 0.55 | 1341.29 ± 104.19 | 50.18 ± 3.90 | 15.00 ± 2.99 | 8.49 ± 0.64 |
| *p-value* | \*\* | \*\*\* | \*\* | NS | NS |
| LSD | 1.83 | 341.54 | 11.87 | 8.8250 | 2.1084 |
| **Asparagus micronutrients reference concentrations (μg g-1 D.W.)** | | | | | |
|  | 40-100 | 40-250 | 25-100 | 20-1000 | 6-12 |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control. Reference values according to Reuter and Robinson (1997)

*3.2. Phenolic compounds in asparagus leaves and rhizomes*

In previous experiments, a higher accumulation of certain phenolic compounds was observed in old asparagus residues in the field and these compounds are thought to contribute to asparagus decline (Elmer, 2018). In the present experiment, we found significant differences in the concentration of compounds derived from caffeic acid (caffeoyl hexose I), presenting higher concentrations the asparagus leaves from the plot with decline. Indeed, this compound was not detected in asparagus leaves from the plot without decline. On the other hand, considering leaf flavonoids, the leaves from the plots with decline showed lower vicenin-2 and kaempferol concentrations. Rutin (quercetin-3-O-rutinoside) concentration was not significantly different between plots, even though the contents were higher in the plot with decline. Fuentes-Alventosa et al. (2008) reported that the flavonoid profile of commercial varieties of green asparagus contains rutin as the main flavonoid, representing approximately 90% of the total flavonoids content. In addition, asparagus possess up to eight different flavonoids glycosides derived from three different aglycones (quercetin, kaempferol, and isorhamnetin). These results were coincident with the data of this experiment: in the plot without decline, rutin represented 84% of the total phenolics, whereas in the plot with decline this percentage was even higher (93%).

**Table 3. Phenolic compounds concentration in asparagus leaves and rhizomes**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Phenolic acids (mg g-1 DW)** | | | |  | **Flavonoids (mg g-1 DW)** | | | |
|  | **Caffeic acid derivatives** | **Caffeoyl-hexose I** | **Caffeoyl hexose II** | **Caffeic acid** |  | **Vicenin-2** | **Quercetin-rut. (Rutin)** | **Kaempferol-rut.** | **Isorhmanetin-rut.** |
| **Leaves** |  |  |  |  |  |  |  |  |  |
| Without decline | 0.68 ± 0.01 | ND | 0.35 ± 0.01 | ND |  | 2.05 ± 0.03 | 15.01 ± 0.17 | 0.36 ± 0.01 | 0.53 ± 0.01 |
| Decline | 1.68 ± 0.09 | 0.15 ± 0.01 | 0.38 ± 0.02 | ND |  | 0.63 ± 0.03 | 16.51 ±0.65 | 0.24 ± 0.01 | 0.49 ± 0.02 |
| *p-value* | \*\*\* | \*\*\* | NS |  |  | \*\*\* | NS | \*\*\* | NS |
| LSD | 0.26 | 0.02 | 0.07 |  |  | 0.13 | 1.85 | 0.03 | 0.06 |
| **Rhizomes** |  |  |  |  |  |  |  |  |  |
| Without decline | ND | 0.11 ± 0.01 | 0.24 ± 0.02 | 0.26 ± 0.01 |  | ND | ND | ND | ND |
| Decline | ND | 0.08 ± 0.01 | 0.140 ± 0.001 | 3.16 ± 0.15 |  | ND | ND | ND | ND |
|  |  | NS | \*\* | \*\*\* |  |  |  |  |  |
| LSD |  | 0.03 | 0.05 | 0.42 |  |  |  |  |  |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control. Rut.=rutinoside

Table 3 shows the phenolic compounds present in asparagus rhizomes and caffeoyl- and caffeic- derivatives presented significant differences between plots. No flavonoids were detected in rhizomes and these results were also found by Symes et al. (2018) where the presence of flavonoids, especially rutin, was not detected in different asparagus rhizome extracts. This could be due to the conditions of sample preparation, by the degradation of the rutin when cutting the shoots. Regarding caffeic acid, it is a hydrocinnamic acid that contributes to autotoxicity in asparagus rhizomes (Symes et al., 2018). The present study results showed that caffeic acid concentration in rhizomes in the plot with decline was 1115% higher than in the plot without decline. Indeed, comparing leaves and rhizomes, we observed that the highest phenolic acid concentrations were present in rhizomes. Consequently, the effect of phenolic acids on autotoxicity might be mainly produced in rhizomes due to the higher caffeic acid accumulation.

*3.3. Quality parameters of the asparagus spear*

In asparagus cultivation, both crop quality and yield decrease after successive years producing the asparagus decline issue (Yeasmin et al., 2014; Kato-Noguchi et al., 2018). This issue causes that asparagus culture becomes unprofitable to maintain due to the reductions in plant density, in the productivity per plant, and because of the smaller spears (Elmer 2018). In the present study, table 4 shows the results of spear weight, diameter, and length comparing spears with and without decline. Regarding length, no significant differences were found which is attributed to the fact that producers ensure the uniformity of spear size for marketing. Based on asparagus classification standards of the European Union (Commission of the European Communities, 1999), the spears collected in the plot without decline can be classified as class I, because the average length was 10.33 mm. In contrast, the spears from the plot with decline were class II with an average length of 9.56 mm. Regarding weight, the spears from the plot without decline presented 22% more weight than the spears from the plot with decline. Siomos (2018) reported the results of several authors for these classification parameters in 29 cultivars of asparagus and the average range spear diameter was 12 to 17 mm. Likewise, the weight had an average of 17.2 to 27.0 g in seven cultivars of 5 production years. Therefore, our results coincide with those reported. In addition, if the loss percentage of spears from plots with decline were multiplied by the production reported by the FAO(2018), the loss would be 11.97 tons in Spain and 6.05 tons in Granada, according to the production reported by the Junta de Andalucía in 2015 (Consejería de Agricultura, Pesca y Desarrollo Rural, 2015).

**Table 4. Classification parameters for asparagus spear quality**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | | |
|  | **Weight (g)** | **Diameter (mm)** | **Length (cm)** |
| Without decline | 20.75 ± 1.16 | 10.33 ± 0.78 | 20.56 ± 0.95 |
| Decline | 16.21 ± 1.69 | 9.56 ± 0.53 | 20.72 ± 0.98 |
| *p-value* | \* | NS | NS |
| LSD | 3.62 | 2.00 | 2.89 |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control.

Another factor that determines food quality is pigment concentration. Thus, previous studies indicated that photosynthetic pigments have benefits for human health. Chlorophylls besides being crucial in photosynthesis, have antioxidant, anti-carcinogenic, anti-inflammatory, anti-obesity and neuroprotective properties (Duppeti et al., 2017). In table 5, it can be observed that chlorophylls a and b were found in greater concentration in plants from the plot with decline, although only significant differences are found in chlorophyll a. In addition, there is evidence that carotenoids are antioxidants that protect cells against reactive oxygen species and free radicals, therefore, they have powerful health benefits (Paznocht et al., 2018). Our results revealed significant differences in carotenoids concentration, being higher in the spears of the plot with decline. In general, the photosynthetic pigments in the spears were more concentrated in the plot with decline. From the nutritional point of view, this may represent an advantage to obtain spear with a higher concentration of these compounds.

**Table 5. - Photosynthetic pigments contents in asparagus spear**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Photosynthetic pigments (mg g-1 D.W.)** | | |
| **Spear** | **Chlorophyll a** | **Chlorophyll b** | **Carotenoids** |
| Without decline | 0.0412 ± 0.01 | 0.0347 ± 0.0006 | 45.79 ± 0.90 |
| Decline | 0.0447 ± 0.01 | 0.0352 ± 0.0003 | 47.89 ± 0.36 |
| *p-value* | \*\* | NS | \* |
| LSD | 0.01 | 0.01 | 2.06 |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control.

Likewise, phenolic compounds found in asparagus contribute to increase the antioxidant capacity of this food (Siomos, 2018). Table 6 shows the results for phenolic concentrations in spears. Spears from the plot with decline presented lower contents of 3-CQA and feruloyl-hexose. On the other hand, only feruloyl-quinic-acid concentration was significantly higher in spears from the plot with decline. Fuentes-Alventosa et al. (2007)reported that the main flavonoid glycosides found in different asparagus varieties were rutin, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside. This finding is in agreement with the content of flavonoids in both plots studied. In addition, regarding flavonols, significant differences were found in quercetin-rutinoside-hexoside 1 and rutin being higher in plots without decline by 17% and 25%, respectively. This result can be considered as an advantage for an added value in the product because rutin is very relevant as a health promoter, and the most abundant flavonol in green asparagus (Motoki et al., 2012). According to our results, the rutin content in the spears without decline represented 90% of the total phenolics, whereas in the spears with decline rutin concentration was lower (85%). Thus, spears from the plot with decline registered a lower content of phenolic compounds. Given the importance that these compounds currently have, this could cause a disadvantage in the market for the spears from the plots with decline due to their lower concentrations. Likewise, the results of phenolic compounds showed that although decline may increase phenols in rhizomes and leaves, these compounds do not accumulate in the spears.

**Table 6.** **– Phenolic compounds present in asparagus spear**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **3-CQA** | **p-Coumaroyl-hexose** | **Feruloyl-QA** | **Feruloyl-hex** | **Q-Rut-hex I** | **Q-rut (Rutin)** | **Q-Rut-hex II** | **Kaempferol rut** | **Isorhamnetin rut** | **Total concentration** |
| **mg g-1 D.W.** | | | | | | | | |  |
| Without decline | 0.68 ± 0.03 | 0.09 ± 0.01 | 0.27 ± 0.02 | 0.09 ± 0.01 | 0.35 ± 0.01 | 5.02 ± 0.26 | 0.060 ± 0.001 | 0.08 ± 0.01 | 0.05 ± 0.01 | 6.69 ± 0.36 |
| Decline | 0.44 ± 0.01 | 0.08 ± 0.01 | 0.56 ± 0.03 | 0.01 ± 0.01 | 0.49 ± 0.01 | 3.79 ± 0.15 | 0.050 ± 0.001 | 0.08 ± 0.01 | 0.07 ± 0.01 | 5.57 ± 0.23 |
| *p-value* | \*\* | NS | \*\* | \*\*\* | \*\*\* | \* | \* | NS | NS | \*\*\* |
| LSD | 0.09 | 0.02 | 0.10 | 0.01 | 0.02 | 0.83 | 0.00 | 0.01 | 0.02 | 0.30 |

The values ​​are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control. 3-CQA= 3-Caffeoyl-QA; QA=quinic acid; hex= hexose; Q=Quercetin, rut=rutinoside,

*3.4. Antioxidant capacity tests*

There are different tests to evaluate the antioxidant capacity in foods and plant extracts, which can be classified based on the reaction mechanism that the antioxidants have to stabilize free radicals (Rosales et al., 2011). In this study we used the DPPH, reducing power, FRAP and TEAC tests. These tests measure the ability to reduce pro-oxidant substances by using different ionic radicals or methyls as oxidants (Sánchez-Rodríguez, et al., 2010). Siomos (2018) reported that antioxidant tests and phenolic compound's results vary depending on factors such as the asparagus harvest. Figure 1A shows the results of the DPPH measurement. Plants from the plots with decline showed 20% higher DPPH value in comparison to spears without decline. Siomos (2018) indicated that DPPH values and other tests in spears can be affected by the harvest period. However, the phenols concentration was not affected. These results agree with our results since the phenolic compounds were lower in spears of the plots with decline (Table 6) while the DPPH levels were higher (Figure 1A). Concerning reducing power and TEAC results no significant differences were found between the spears from both plots, however, the values were greater in the plot with decline (Figure 1B and 1C). On the other hand, the FRAP test did show significant differences being 6.3% higher in the spears from the plot with decline (Figure 1D). Therefore, these results indicate that the spears from the plot with decline possess a greater antioxidant capacity, possibly because the plants are more stressed.



**Figure 1.** Results of the antioxidant tests in asparagus spears from plots with and without decline, (A) DPPH, (B) reducing power, (C) TEAC, (D) FRAP. The values are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control.

Ascorbate is an important phytonutrient that is directly involved in ROS elimination and presents antioxidant properties when is consumed by humans (Rosales et al., 2011). In addition, Matsubara et al. 2010 observed in a study that ascorbate could be important to counteract decline effects in mycorrized asparagus. Figure 2 shows the results of reduced ascorbate. The spears with decline showed a higher concentration than the spears of the plot without decline. Therefore, the overall results of the antioxidant tests and the reduced ascorbate concentration may indicate that the spear from the plot with decline presented more stress. This could be an advantage from the nutritional point of view because they would have more antioxidant compounds. However, from the agronomic point of view, stress can cause large losses in asparagus production volume as it was indicated above.



**Figure 2.** Reduced ascorbate concentration in asparagus spears from plots with and without decline. The values are means ± standard error (n = 9) and the differences between means were compared with Fisher's Minimum Significant Difference test (LSD, P = 0.05). Significance levels are represented by NS (not significant) P> 0.05; \* P <0.05; \*\* P <0.01 and \*\*\* P <0.001 with respect to the control. Different letters mean different groups.

*3.5. Toxicity tests*

Asparagus autotoxicity is thought to be a factor to explain asparagus decline. Indeed, some studies observed that some autotoxic compounds released by asparagus plants possibly inhibit the growth of asparagus and other species seedlings causing the replanting issue (Yeasmin et al., 2014; Kato-Noguchi et al., 2018). To verify this effect in the plots of the present study, we measured germination percentage and epicotyl growth of seeds that received leaf extracts from both plots (Figures 3 and 5). The leaf extracts from the plot without decline caused a similar decrease in the germination percentage regardless of the dose applied. However, seeds that received 150 µl and 250 µl of the leaf extract from the decline plot showed lower germination than the seeds treated with leaf extracts from the plot without decline. Figure 3B presents the epicotyl growth in the treatments. The 150 μl and 250 μl doses of leaf extracts from the plot without decline showed significant differences compared to control. Nevertheless, all the doses of the leaf extracts from the plot with decline registered significant differences.



**Figure 3.** Effect of different doses of foliar extracts from the plots with and without decline on the number of germinated seeds (A) and the epicotyl growth (B). Values are expressed as means ± standard error (n=9). The columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

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**Figure 4.** Images showing the effect of leaf extracts from plants without decline (A) and with decline (B) on the germination of lettuce seeds.

Figures 5A and 6 show the germination percentage of seeds that received rhizomes extracts from both plots. In the extracts from the plot without decline only the 150 μl and 250 μl doses showed significant differences compared to controls, whereas in the seeds that received the decline plot extracts all doses were significantly different in comparison to controls. Figure 5B shows the effect of the rhizomes extracts on epicotyl growth. Regarding the plot without decline the 50 μl and 150 μl doses caused significant differences compared to controls, showing total absence of growth in the seeds with 250 μl dose. In contrast, the 50 μl dose extracts from the plot with decline produced significant differences and the seeds that received 150 μl and 250 μl doses did not show growth. Similar results were observed by Kato-Noguchi (2017; 2018) in other experiments that applied methanol extracts from a culture of asparagus with decline. These extracts inhibited the growth of seedlings of asparagus and other species in a concentration-dependent manner. Therefore, in the present experiment, the negative effects on germination and epicotyl growth could be attributed to the fact that compounds of asparagus extracts produced phytotoxic effects. These effects had a greater impact when extracts come from both leaves and rhizomes of the plot with decline. In addition, it is noteworthy that the rhizome extracts had a greater phytotoxic effect than the leaf extracts, preventing epicotyl growth at the highest doses.



**Figure 5.** Effect of different doses of rhizome extracts from the plots with and without decline on the number of germinated seeds (A) and the epicotyl growth (B). Values are expressed as means ± standard error (n=9). The columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

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**Figure 6.** Images showing the effect of rhizome extracts from plants without decline (A) and with decline (B) on the germination of lettuce seeds.

**4. Conclusion**

We conclude that, in our experiment, the decline did not cause great changes in macronutrient concentration. However, it possibly caused B deficiency and Fe toxicity that might contribute to increase decline symptoms. On the other hand, the concentration of phenolic compounds was greater in the plot with decline in leaves and rhizomes but not in spears. In rhizomes, it highlights the great accumulation of caffeic acid which could be a decline indicator. Likewise, the decline decreases the market spear quality reducing their sizes and weight, which could cause great economic losses. However, decline slightly increased some quality indicators such as chlorophylls, carotenoids, ascorbate and antioxidant capacity which was associated with higher stress. On the other hand, the negative effects of asparagus extracts from the decline plot on seed germination and epicotyl growth suggest a potential allelopathic effect on future cultures. In short, the results obtained in this study are useful to provide a better understanding of the effects of decline issue in asparagus. In addition, it provides information for asparagus producers about the effect of decline on asparagus market quality and its allelopathic effect.

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**References**

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Cai, Y., Luo, M., Sun, H., & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medical plants associated with anticancer. *Life Sciences*, *74*, 2157–2184.

Commission of the European Communities (2019). Commission regulation (EC) No 2377/1999 of 9 November 1999 laying down the marketing standard for asparagus. Official Journal of the European Communities.

Consejería de agricultura, pesca y desarrollo rural. Anuario de estadísticas agrarias y pesqueras Junta de Andalucia (2015). URL (<http://www.juntadeandalucia.es/organismos/agriculturapescaydesarrollorural/consejeria/sobre-consejeria/estadisticas/paginas/agrarias-anuario.html>) (05.05.2018)

Duppeti, H., Chakraborty, S., Das, B. S., Mallick, N., & Kotamreddy, J. N. R. (2017). Rapid assessment of algal biomass and pigment contents using diffuse reflectance spectroscopy and chemometrics. *Algal Research*, *27*, 274–285.

Elmer, W. (2018). Asparagus decline and replant problem: A look back and a look forward at strategies for mitigating losses. In *XIV International Asparagus Symposium* 1223 (pp. 195-204).

Elmer, W. H., & Pignatello, J. J. (2011). Effect of biochar amendments on mycorrhizal associations and Fusarium crown and root rot of asparagus in replant soils. *Plant Disease*, *95*, 960–966.

FAO (Food and Agriculture Organization of the United Nations) (1991). Guía para la manipulación de semillas forestales.

FAO, Food and Agriculture Organization of the United Nations (2018). FAOSTAT. URL (http://www.fao.org/faostat/es/#data/QC) (05.06.18).

Fuentes-Alventosa, J. M., Rodríguez, G., Cermeño, P., Jiménez, A., Guillén, R., Fernández-Bolaños, J., & Rodríguez-Arcos, R. (2007). Identification of flavonoid diglycosides in several genotypes of asparagus from the Huétor-Tájar population variety. ‎*Journal of Agricultural and Food Chemistry*, *55*(24), 10028–10035.

Fuentes-Alventosa, J. M., Jaramillo, S., Rodríguez-Gutiérrez, G., Rodríguez-Gutiérrez, G., Espejo, J. A., Jiménez-Araujo, A., Guillén-Bejarano, R., Fernández-Bolaños, J., & Rodríguez-Arcos, R. (2008). Flavonoid profile of green asparagus genotypes. *Journal of Agricultural and Food Chemistry*, *56*, 6977–6984.

Hsu, C., Chen, W., Weng, Y., & Tseng, C. (2003). Chemical composition, physical properties, and antioxidant activities of yam flours as affected by different dryingmethods. *Food Chemistry*, *83*, 85–92.

Kato-Noguchi, H., Nakamura, K., Ohno, O., Suenaga, K., & Okuda, N. (2017). Asparagus decline: Autotoxicity and autotoxic compounds in asparagus rhizomes. *Journal of Plant Physiology*, *213*, 23–29.

Kato-Noguchi, H., Nakamura, K., & Okuda, N. (2018). Involvement of an autotoxic compound in asparagus decline. *Journal of Plant Physiology*, *224*, 49–55.

Krom, M. D. (1980). Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. *Analyst*, *105*, 305–316.

Law, M. Y., Charles, S. A., & Halliwell, B. (1992). Glutathione and ascorbic acid in spinach (*Spinacea oleracea*) chloroplast: the effect of hydrogen peroxide and paraquat, *Biochemical Journal*, *210*, 899–903.

Lu, H., Zheng, H., Lou, H., Jiang, L., Chen, Y., & Fang, S. (2010). Using neural networks to estimate the losses of ascorbic acid, total phenols, flavonoid, and antioxidant activity in asparagus during thermal treatments. *Journal of Agricultural and Food Chemistry*, *58*(5), 2995–3001.

Marschner, H. (2011). Marschner’s Mineral Nutrition of Higher Plants, Petra Mars, Academic Press.

Matsubara, Y., Okada, T., & Nahiyan, A. S. M. (2009). Tolerance to allelopathy and Fusarium disease, changes in antioxidative substances in mycorrhizal asparagus plants raised in decline soil. In *VII International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation*, 883 (pp. 417-423)

Miller, H. G., Ikawa, M., & Peirce, L. C. (1991). Caffeic acid identified as an inhibitory compound in asparagus root filtrate. *HortScience*, *26*(12), 1525–1527.

Motoki, S., Kitazawa, H., Kawabata, T., Sakai, H., Matsushima, K.I., & Hamauzu, Y. (2012). Rapid rutin accumulation during spear elongation in asparagus. *HortScience*, *47*(5), 599–602.

Paznocht, L., Kotíková, Z., Šulc, M., Lachman, J., Orsák, M., Eliášová, M., & Martinek, P. (2018). Free and esterified carotenoids in pigmented wheat, tritordeum and barley grains. *Food chemistry*, *240*, 670–678.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. 1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, *26*, 1231–1237.

Reuter, D., & Robinson J. B. (Eds.) (1997). Plant analysis: an interpretation manual. CSIRO publishing.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández-Bolaños, J., & Jiménez, A. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. *Journal of Agricultural and Food Chemistry*, *53*(13), 5212–5217.

Rosales, M. A., Cervilla, L. M., Sánchez‐Rodríguez, E., Rubio‐Wilhelmi, M. D. M., Blasco, B., Ríos, J. J., & Ruiz, J. M. (2011). The effect of environmental conditions on nutritional quality of cherry tomato fruits: evaluation of two experimental Mediterranean greenhouses. *Journal of the Science of Food and Agriculture*, *91*(1), 152-162.

Sanae, M., & Yasuo, A. (2013). Green asparagus (*Asparagus officinalis*) prevented hypertension by an inhibitory effect on angiotensin-converting enzyme activity in the kidney of spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry*, *61*(23), 5520–5525.

Sánchez-Rodríguez, E., Rubio-Wilhelmi, M., Cervilla, L. M., Blasco, B., Rios, J. J., Rosales, M. A., Ruiz, J. M. (2010). Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Science*, *178*(1), 30-40.

Siomos, A. S. (2018). The quality of asparagus as affected by preharvest factors. *Scientia Horticulturae*, *233*, 510-519.

Symes, A., Shavandi, A., Zhang, H., Mohamed Ahmed, I. A., Al-Juhaimi, F. Y., & Bekhit, A. E. D. A. (2018). Antioxidant activities and caffeic acid content in New Zealand asparagus (*Asparagus officinalis*) roots extracts. *Antioxidants*, *7*(4), 52.

Webber III, C. L., White Jr, P. M., Landrum, D. S., Spaunhorst, D. J., Wayment, D. G., & Dorvil, E. (2017). Sugarcane field residue and root allelopathic impact on weed seed germination. *Journal of Agricultural Science*, *10*(1), 66.

Wellburn, A. R. (1994). The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*, *144*, 307–313.

Wolf, B. (1982). A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. *Communications in Soil Science and Plant Analysis*, *13*, 1035–1059.

Yeasmin, R., Nakamatsu, K., Matsumoto, H., Motoki, S., Nishihara, E., & Yamamoto S. (2014). Inference of allelopathy and autotoxicity to varietal resistance fasparagus (*Asparagus officinalis* L.). *Australian Journal of Crop Science*, *8*, 251–25