
*International Journal of Plant & Soil Science*

***Volume 35, Issue 1, Page 47-61, 2023; Article no.IJPSS.95934***
***ISSN: 2320-7035***

# Rhizobacteria-Based Biostimulant Mixture Effect on Chickpea (*Cicer arietinum* L.) in Greenhouse and Cultivation Assays

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/IJPSS/2023/v35i12725

**Open Peer Review History:**
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/95934

***Original Research Article***

***Received: 22/11/2022***
***Accepted: 28/01/2023***
***Published: 01/02/2023***

## ABSTRACT

This study aims to contribute to the improvement of the chickpea agri-food industry and adapt it to future challenges. For this, the effect of a Rhizobacteria-based biostimulant mixture was analyzed in Amelia variety of chickpea (*Cicer arietinum* L.) in two different experiments: one in flowerpots in a greenhouse and the other one in the field grown under rainfed conditions. Germination success, vegetative development and phenological stages of the plants were studied after applying liquid

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biostimulant mixture on the seeds 24 h before sowing or spraying it on the soil after sowing. The results indicated that the way of application of the biostimulant was relevant concerning germination success, vegetative development, and phenological state of chickpea plants and it should be adapted to intensive agriculture with the use of seed drill that requires dry seeds. Regarding germination success, vegetative development and phenological stages in the flower pot assay, the volume of biostimulant mixture applied to the seeds was remarkable as higher volumes affected negatively while lower volumes were positive. Biostimulant had a negative effect on the development of chickpea plants in the field assay, probably because it also promoted the growth of weeds, which lessened the growth of the chickpea plants.

*Keywords: Chickpea; biostimulant; Cicer arietinum; weeds.*

## 1. INTRODUCTION

To improve the efficiency of the chickpea agri-food industry and adapt it to climate change, our group is studying the effect of biostimulants on chickpea (*Cicer arietinum* L.) cultivation. Because of climate change, the average annual temperature of the agricultural year and the corresponding to summer months have increased; while rainfall recorded during the month of May, as well as the period of frosts, have decreased. Chickpea yields are harmed both in conventional and ecological management due to climate change, especially because of the decrease in rainfall, in particular the corresponding to the month of May [1] when the reproductive stages of the plant develop. Agricultural practices that anticipate the reproductive stages of chickpea plants to avoid their coincidence with periods with high temperatures and hydric stress could be an adaptation to climate change; i.e. the development of chickpea varieties with an earlier date of sowing, reproductive stages and harvest season or the use of biostimulants that accelerate and improve the vegetative development of the plants.

There are different types of biostimulants: based on bacteria and fungi, algae products, and chitosan products, among others [2]. Among the ones based on beneficial microorganisms, the group called "Plant Growth-Promoting Bacteria" (PGPB) is one of the most used treatments in chickpea [3]. One of the most common genera of PGPB is *Rhizobium* sp. Indeed, biostimulants with *Rhizobium* sp. are called "Plant Growth-Promoting Rhizobacteria" (PGPR). *Rhizobium* sp. is a genus of atmospheric nitrogen-fixing microorganisms that form mycorrhizae with the chickpea root and incorporate nitrogen into the plant and the soil [4]. Therefore, sometimes there are no differences in production between fertilized and unfertilized plots [4]. *Rhizobium* sp. can replace 50-100% of N fertilizers [2] during the final phases of cultivation.

A lot of variables have been analyzed in chickpea cultivations in which biostimulants had been applied with beneficial microorganisms: nodulation, growth (height, diameter, total plant mass, dry mass of nodules, leaf area index, greenness index, volatile compounds), yield (number of pods, pod mass, number of grains, mass of grains, grain protein content, biofortification), germination (percentage and date after the sowing), sprout and root length, N, P and K uptake; and also the incidence of several diseases and pests, etc. [2]. All these variables showed positive effects of biostimulants that contain beneficial microorganisms on chickpea cultivation [2]: i.e. greater formation of mycorrhizae nodules, plant growth and harvest [5-9], higher protein content in the grain [6,10], biofortification [10]; more absorption of N, P, K; increased activity of the enzymes SOD (superoxide dismutase) and POD (peroxidase); and an increase in the concentrations of organic acids, thus reducing the pH of the rhizosphere [11]. In addition and as we said previously, infections by phytopathogens were inhibited or reduced [12-14]. Moreover, PGPB respect the environment and require few economic costs for their production, which reduces the use of non-renewable sources [15], the degradation and contamination of the soil produced by agrochemicals avoided and it contributes to the restoration of the soil microbial balance and abiotic stress is reduced [2].

Regulation (EU) 2019/1009 [16] defines the term biostimulant as “an EU fertilizer product whose function is to stimulate the nutritional processes of plants regardless of the nutrient content of the product, with the sole objective of improving one or more of the following plant characteristics and its rhizosphere: efficiency in the use of nutrients, tolerance to abiotic stress, quality characteristics or availability of nutrients immobilized in the soil and the rhizosphere". Regulation (EU) 2019/1009 [16] also establishes that biostimulant developing companies may only declare on the label those benefits of the product that have been agronomically tested on the crops through an exhaustive and standardized control efficacy tests that verifies and accredits, through an audit by a third company, the biostimulant action that is declared on the label. The new legislative framework differentiates two types of biostimulants: microbial (CFP 6, A) and non-microbial (CFP 6, B). Within the first type, the European regulation only allows the use of four microorganisms to formulate biostimulants: *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp. and *Azospirillum* spp. In Spain, legislation requires the presentation of extensive documentation endorsed by an independent entity that certifies the efficacy, characteristics and safety of products based on microorganisms (RD 999/2017) [17].

The biostimulant used in the present research was a mixture of beneficial microorganisms, algae-based products, vitamins, amino acids, and other products. The variety of chickpea used was Amelia, a Desi-type that has been improved through different research projects in Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA). It is well-known and marketed in the agricultural sector and it has high performance (kg/ha).

We analyzed the germination success (percentage and date after the sowing), vegetative development (number of nodes, plant length and diameter) and phenological state of the plants when the biostimulant mixture was applied liquid on the seeds 24 h before sowing or sprayed on the soil just after the sowing. Two different experiments were performed, one in a greenhouse in flower pots and another one in the field, grown under rainfed conditions.

## 2. MATERIALS AND METHODS

Biostimulant used in both experiments was composed of a powder base that contained mainly PGPB rhizobacteria (*Azospirillum brasilense*, *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens*), endomycorrhizal fungi (*Glomus intraradices*, *Trichodema harzianum*, *T. reesei*, *T. viride*, and *Gliocladium virens*), vitamins (biotin, folic acid, B, B2, B3, B6, B7, B12, C, and K), amino acids, soluble extract of *Yucca schidigera* and *Ascophyllum nodosum*, and a treacle base obtained from plant extracts used as a diluent.

### 2.1 Flowerpot Assay

Liquid biostimulant was applied to 21 samples of Amelia chickpea grains in three concentrations: 0 (control), 1:10 and 1:5 (1 dose of biostimulant and the rest of distilled water). The biostimulant was applied liquid to the seeds 24h before sowing or sprayed on the soil just after the sowing. When biostimulant was added liquid to 100 g of seeds, dif-ferent volumes were applied: 2.5, 5 and 10 ml. When it was applied in the form of spray on the soil, the numbers of spray applications were: 0, 1, 3 and 5.

After the samples of 100g of chickpea grains were soaked into the liquid of the different treatments, they were shaken 20 times. Then, they were removed to Petri dishes to dry and avoid excess moisture. Five replicates of each treatment were performed. Finally, for the length measurements of the plants, two replicates were done.

Already in the greenhouse, a series of pots 11cm x 11cm and 14 cm deep were arranged with substratum mixed with 10% perlite. A first abundant irrigation was carried out one hour before sowing, on January 27, 2022. Subsequently, one chickpea seed per pot was placed one centimeter deep and covered with the substratum and perlite mixture.

Although the chickpea is a rainfed crop, given the growth conditions in pots and a greenhouse, it was necessary to irrigate. 55 mL of water were added periodically. The pots were protected with mesh to prevent damage from birds or other animals.

Parameters evaluated were the germination success (percentage), vegetative development (plant length and node number) and phenological evolution of the crop, following the Schwartz and Langham scale [18]. Data collection of these variables was carried out every 3-4 days, using a metric ruler.

### 2.2 Field Assay

The experiment was carried out in two land plots at El Encín, belonging to IMIDRA, located in the municipality of Alcalá de Henares, Madrid, Spain. The area of each plot was 20m x 60m and they were surrounded by electric wiring. Previous to the sowing, the soil was removed with a cropper. The biostimulant mixture was made at the rate of one part of a powder base and one part of a treacle base. This mixture was diluted at a rate of one part per nine of water; and 25ml of this broth was applied to one kilogram of chickpea seeds. The mixture was applied directly to the seed 24 h before sowing using a small concrete mixer to ensure that the entire batch was impregnated. Sowing was carried out with a conventional grain seeder calibrated at a dose of 125-145 kg/ha on April 8, 2022. The sowing frame in all of them was 3 passes using 4 boots and then 2 passes using 6 boots (3 groups of 2), in the Northwest direction. In the configuration with 4 boots, the dose was 125 kg/ha with the variator at position 90 and the flap opening at position 7; while in the configuration with 6 boots it was 145 kg/ha with the variator at position 46 and the flap opening to position 7. Three herbicides were applied in the land plots: on May 5, 2022, tquizalofop-p-ethyl 5% w/v at a dose of 2 L/ha, and pendimethaline 40% w/v at a dose of 5 L/ha, and, on May 11, 2022, glyphosate along the perimeter of the plot. On May 19, June 8 and 22, 2022, weeds were removed with a cropper.

Parameters evaluated were the phenological evolution of the crop, following the BBCH scale [19] and four quantitative variables of the vegetative development: plant length and diameter, pod and seed length in centimeters (cm). Data collection of these variables was carried out on the 16th of June, 2022, using a metric ruler, from three chick-pea plants chosen at random by treatment; while monitoring of the phenological state was done throughout the entire growth period of the plant once every week.

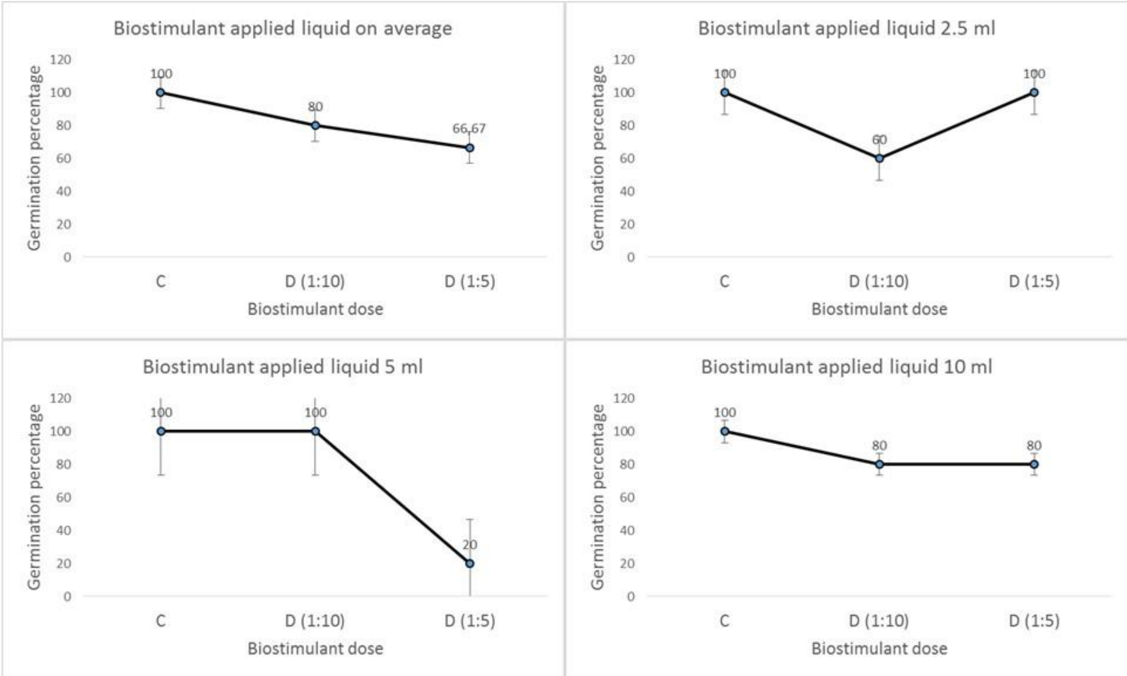
Statistical data processing was performed using: the IBM 26th SPSS Statistics version computer tool and the following parameters were evaluated: height, seed length, canopy, and pod length, in centimeters (cm). From them, the mean value and standard deviation were calculated, and a simple ANOVA analysis of variance was performed, with a significance level of 95%. Kruskal-Wallis test and Dunn's Multiple Comparison Test were performed with the software GraphPad Prism 5. Correspondence analysis with the weeds was realized with the program Rstudio, version 1.4.1717.

## 3. RESULTS

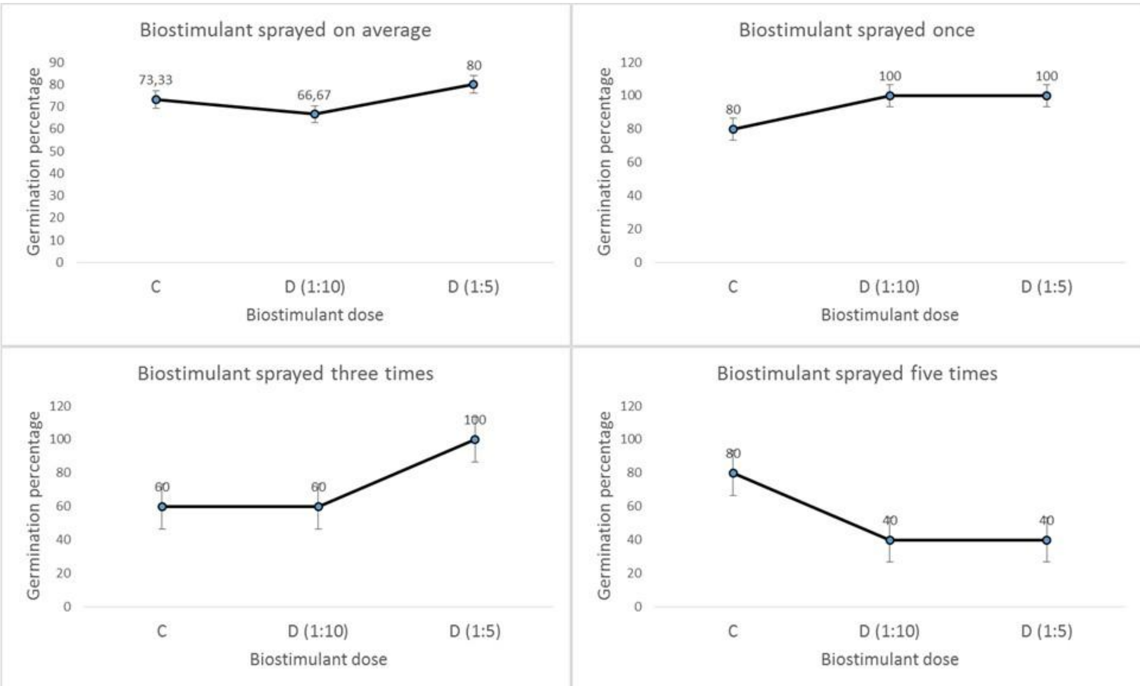
### 3.1 Flowerpot Assay

14 days after the sowing, emergence was observed in all the treatments. The percentage of germination showed that lower volumes of biostimulant were a positive influence on germination success while higher volumes resulted negative for germination (Fig. 1). When 100 g of seeds were applied with small volumes of biostimulant 24 h before the sowing or biostimulant was sprayed on the soil once or three times just after the sowing, then biostimulant influenced positively germination success and the higher the dose the higher the germination success. On the contrary, there was a negative tendency when the volume applied was higher: 5 and 10 ml to 100 g of seeds 24 h before the sowing or 5 spray applications to the soil just after the sowing. The results indicated that 100 g chick-pea seeds should be applied in small volumes of biostimulant, 2.5 ml or less, 24 h before sowing, or sprayed 1-3 times on the soil just after the sowing, to obtain optimum germination success. According to the Kruskal-Wallis test the medians of the results of the different treatments vary significantly (P value 0.0160) but Dunn's Multiple Comparison Test doesn't show any significant difference in rank sum when it compared treatments in pairs.

The phenological study of the chickpea plants on pots showed similar results (Fig. 2, Table 1): Lower quantities of liquid biostimulant (2.5 ml) or sprayed once, promoted a positive effect on plant growth; plants grew more on average with 1:5 doses than with 1:10 and control. On the contrary, when more quantity of liquid biostimulant (5-10 ml) was used to treat the seeds or sprayed 3-5 times on the soil, control and lower doses (1:10) showed higher plant growth on average and maximum. There were statistically significant differences among the medians (Kruskal-Wallis statistic 358.3, P value < 0.0001) and between the following pairs of treatments (Dunn's Multiple Comparison Test, Differences in rank sum, Significant $P < 0.05$): Control 2.5 ml vs D (1:10) 2.5 ml, Control 5 ml vs Control 10 ml, D (1:10) 2.5 ml vs D (1:5) 2.5 ml, D (1:5) 2.5 ml vs D (1:5) 10 ml, Control 1 spray vs D (1:10) 1 spray, Control 1 spray vs D (1:5) 1 spray, Control 3 spray vs D (1:10) 3 spray, Control 3 spray vs D (1:5) 3 spray, Control 5 spray vs D (1:10) 5 spray, Control 5 spray vs D (1:5) 5 spray, D (1:10) 1 spray vs D (1:10) 3 spray, D (1:10) 1 spray vs D (1:10) 5 spray, D (1:10) 1 spray vs D (1:5) 1 spray.




(**a**)




(**b**)

**Fig. 1. Germination success graphs in the different treatments of the flowerpot assay: (a) Biostimulant applied to 100 g of chickpea seeds 24 h before sowing, on average and when 2.5, 5 and 10 ml were applied to 100 g of grains; (b) Biostimulant sprayed on the soil just after the sowing, on average and when it was sprayed once, three and five times to the soil**

**Table 1. Average length of chickpea plants with different treatments on different dates of the culture**

| **Cultivation day** | **29** | **30** | **36** | **37** | **43** | **44** | **50** | **51** | **57** | **58** | **65** | **75** | **82** | **89** | **97** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BioE applied liquid** | | | | | | | | | | | | | | | |
| Control 2.5 ml | 1.5 | | 2.9 | | 4.7 | | 7.0 | | 8.0 | | 8.7 | 8.2 | 9.5 | 9.5 | 9.0 |
| Control 5 ml | 1.7 | | 3.3 | | 5.2 | | 6.8 | | 7.9 | | 8.9 | 8.9 | 8.5 | 8.3 | 9.0 |
| Control 10 ml | 2.2 | | 4.1 | | 6.6 | | 8.4 | | 10.1 | | 11.1 | 11.3 | 11.9 | 12.6 | 13.2 |
| D (1:10) 2.5 ml | 1.6 | | 2.3 | | 3.8 | | 6.2 | | 7.4 | | 11.0 | 8.1 | 8.4 | 8.3 | 8.1 |
| D (1:10) 5 ml | 1.6 | | 3.8 | | 5.8 | | 7.8 | | 9.1 | | 11.2 | 9.9 | 9.8 | 10.5 | 11.3 |
| D (1:10) 10 ml | 3.3 | | 5.4 | | 7.7 | | 10.2 | | 12.0 | | 9.3 | 13.9 | 14.4 | 14.5 | 15.6 |
| D (1:5) 2.5 ml | 2.7 | | 5.1 | | 6.8 | | 9.2 | | 10.4 | | 8.2 | 11.1 | 11.3 | 11.5 | 12.8 |
| D (1:5) 5 ml | 1.3 | | 2.9 | | 5.1 | | 8.4 | | 9.8 | | 9.5 | 10.8 | 11.2 | 12.7 | 14.3 |
| D (1:5) 10 ml | 1.5 | | 3.2 | | 5.1 | | 7.5 | | 8.9 | | 13.4 | 10.0 | 10.3 | 10.0 | 10.5 |
| **BioE sprayed** | | | | | | | | | | | | | | | |
| No treatment 1 | | 3.5 | | 5.2 | | 6.2 | | 9.2 | | 11.1 | 12.3 | 12.6 | 12.4 | 13.8 | 17.5 |
| No treatment 2 | | 10.3 | | 14.5 | | 17.7 | | 20.9 | | 23.7 | 25.4 | 27.4 | 29.4 | 29.4 | 31.0 |
| No treatment 3 | | 6.0 | | 9.2 | | 11.3 | | 13.7 | | 16.3 | 17.3 | 18.9 | 19.3 | 20.6 | 21.9 |
| Control 1 spray | | 4.9 | | 7.8 | | 10.1 | | 12.5 | | 14.1 | 15.0 | 16.0 | 16.0 | 17.9 | 21.4 |
| Control 3 spray | | 4.5 | | 7.5 | | 8.9 | | 11.2 | | 12.9 | 14.6 | 16.1 | 20.3 | 22.7 | 23.3 |
| Control 5 spray | | 7.4 | | 10.2 | | 15.1 | | 17.0 | | 19.6 | 21.9 | 23.5 | 26.5 | 26.6 | 28.5 |
| D (1:10) 1 spray | | 2.1 | | 3.6 | | 5.8 | | 7.9 | | 9.3 | 10.2 | 10.0 | 10.9 | 11.4 | 12.2 |
| D (1:10) 3 spray | | 2.8 | | 4.5 | | 6.7 | | 8.5 | | 10.3 | 11.2 | 11.8 | 12.5 | 13.3 | 13.6 |
| D (1:10) 5 spray | | 1.9 | | 3.9 | | 5.0 | | 6.9 | | 8.9 | 10.0 | 10.1 | 10.2 | 11.2 | 10.4 |
| D (1:5) 1 spray | | 7.7 | | 10.6 | | 12.7 | | 15.8 | | 18.9 | 22.1 | 25.6 | 30.6 | 31.6 | 34.2 |
| D (1:5) 3 spray | | 2.2 | | 3.1 | | 5.2 | | 9.4 | | 10.1 | 11.4 | 11.3 | 11.6 | 11.8 | 11.6 |
| D (1:5) 5 spray | | 2.0 | | 2.5 | | 4.1 | | 6.6 | | 8.4 | 9.6 | 10.3 | 11.7 | 12.5 | 12.6 |

*BioE, biostimulant. Sowing date is cultivation day number 1*

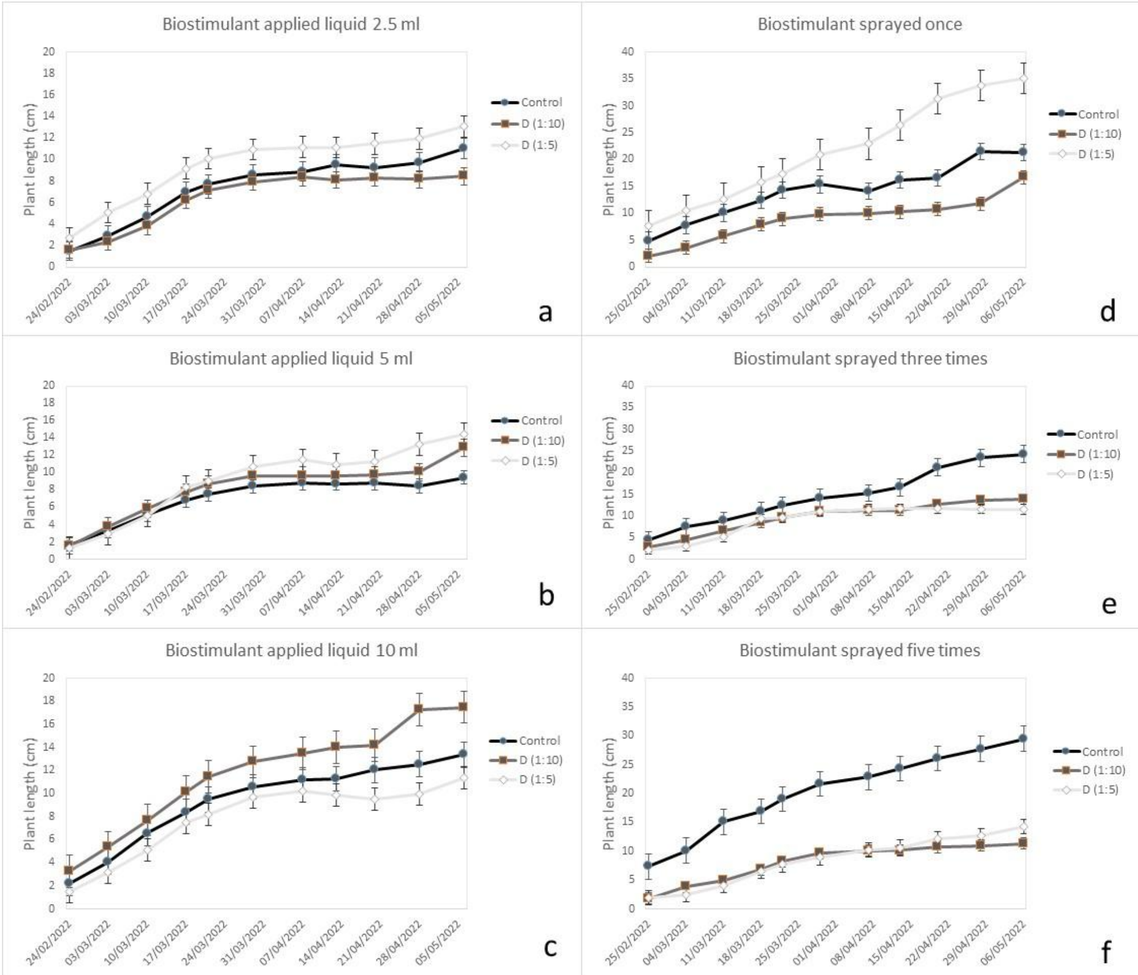



**Fig. 2. Vegetative development on different dates of chickpea plants in the flowerpot assay, indicating plant length (cm): (a) Treatment of 2.5 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (b) Treatment of 5 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (c) Treatment of 10 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (d) Biostimulant sprayed once on the soil just after the sowing; (e) Biostimulant sprayed three times on the soil just after the sowing; (f) Biostimulant sprayed five times on the soil just after the sowing**

The study of the number of nodes in the phenological state of chickpea plants is indicated in Fig. 3, according to Schwartz and Langham scale [18]. The results are similar to plant length.

## 3.2 Field Assay

Amelia is a chickpea variety with high yield (kg/ha), biomass and corrected seed weight (g/m2). Regarding its phenological state without and with biostimulant (Table 2), there were no significant differences on its germination success and its vegetative development. On April 18, 2022, Amelia showed emergence without and with biostimulant, ten days after the sowing.

Remarkably, there were more weeds in the land plot with biostimulant than without biostimulant (Table 2). From June 20, 2022, there was a huge growth of *Amaranthus retro-flexus* L. This lessened the growth of chickpea plants and displaced them to the edges of the land plot.

As indicated in Table 3, length and diameter of plants were larger without biostimulant than with biostimulant, being statistically significantly different in the case of the diameter (simple ANOVA P value 0.0482). The diameter of the seeds was higher without biostimulant, 0.9 cm, than with biostimulant, 0.55 cm. The pod length was the same. Data were collected on June 16, 2022.

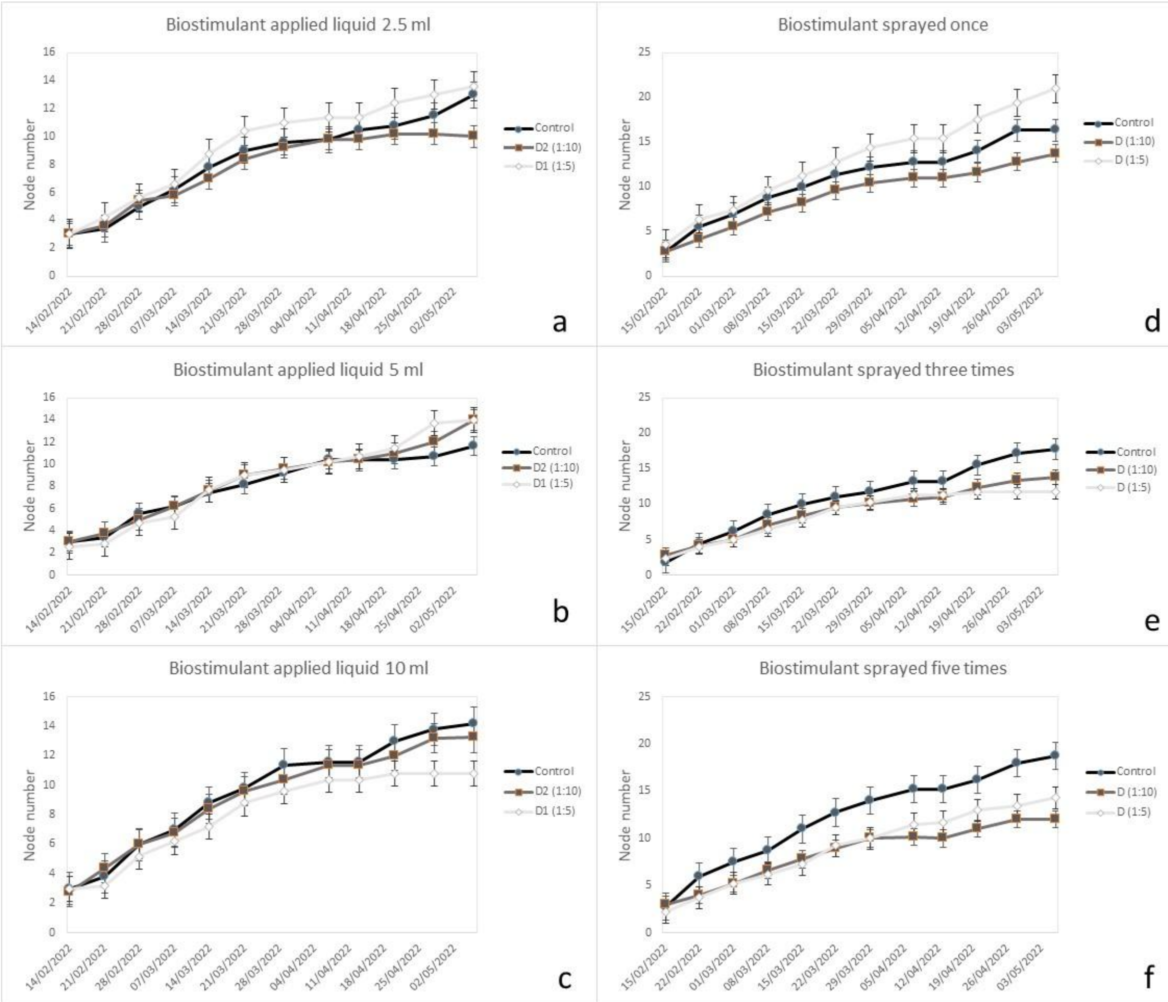



**Fig. 3. Vegetative development on different dates of chickpea plants in the flowerpot assay, indicating the number of nodes following the Schwartz and Langham scale [18]: (a) Treatment of 2.5 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (b) Treatment of 5 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (c) Treatment of 10 ml of biostimulant applied to 100 g of chickpea seeds 24 h before sowing, several biostimulant concentrations; (d) Biostimulant sprayed once on the soil just after the sowing; (e) Biostimulant sprayed three times on the soil just after the sowing; (f) Biostimulant sprayed five times on the soil just after the sowing**

It was performed a correspondence analysis (Rstudio Version 1.4.1717) with the weeds observed in the land plots with Amelia without and with biostimulant (Table 4, Fig. 4).

Species of weeds such as *Sisymbrium officinale* and *Lactuca serriola* were more corre-lated with land plots where Amelia without biostimulant was growth. Species of weeds such as *Dittrichia viscosa*, *Amaranthus retroflexus*, *Datura stramonium* and *Chenopodium vulvaria* were more correlated with land plots where Amelia with biostimulant was present. *A. retroflexus*, *D. stramonium* and *Ch. vulvaria* are more correlated with later dates of observation.

## 4. DISCUSSION

The results indicated that the way of application of the biostimulant was relevant concerning germination success, vegetative development and phenological state of chickpea plants. Higher volumes of liquid biostimulant, and applied on the seeds, affected negatively chickpea germination success. Maybe, the moisture of chickpea grains previous to sowing

**Table 2. Phenological stages of amelia cultivation following the BBCH scale [19]. AM- (amelia without biostimulant), and AM+ (amelia with biostimulant). Observations on different dates**

| Date | AM-stage | Observations AM- | AM+ stage | Observations AM+ |
|---|---|---|---|---|
| 09/05/2022 | 18 | Advanced germination | 17 | Advanced germination |
| 13/05/2022 | 19 | | 19 | |
| 17/05/2022 | 19 | Few weeds | 31 | Abundant weeds, leafminer (*Liriomyza cicerina* Rondani, 1875) |
| 20/05/2022 | 31 | | 31 | |
| 24/05/2022 | 60 | Starts flowering | 60 | Starts flowering |
| 27/05/2022 | 61 | | 61 | |
| 30/05/2022 | 64 | | 64 | |
| 02/06/2022 | 69 | 7-8 flowers/plant | 69 | |
| 06/06/2022 | 71 | > 10 flowers/plant pods | 72 | Pods Abundant weeds |
| 13/06/2022 | 73 | High density of chickpea plants, few weeds, still presence of flowers, 1 seed/pod, high number of pods/plant | 72 | Abundant weeds |
| 16/06/2022 | 74 | Polyphagotarsonemus latus Banks 1904 | 72 | |
| 20/06/2022 | 84 | | 81 | Invasion of *Amaranthus retroflexus* L. in the center of the land plot |
| 24/06/2022 | 86 | Advanced development of the plants, mainly dried plants and grain | 84 | *A. retroflexus* L. occupied the center of the land plot and displaced chickpea plants |
| 27/06/2022 | 87 | | 87 | Huge amount of *A. retroflexus* L. |
| 30/06/2022 | 87 | Still some flowers | 87 | |
| 04/07/2022 | 88 | Superior pods were empty | 88 | Superior pods were empty |
| 08/07/2022 | 89 | Ready for the harvest | 89 | Ready for the harvest |
| 11/07/2022 | 92 | | 91 | |

**Table 3. Average and standard deviation of several magnitudes of chickpea plants. AM- (amelia without biostimulant), and AM+ (amelia with biostimulant). Date of data collection 16/06/2022**

| Parameters | AM - | AM + |
|---|---|---|
| Plant length (cm) | 37.25 ± 2.65 | 32.75 ± 0.25 |
| Plant diameter (cm) | 45.25 ± 2.30 | 27.65 ± 1.66 |
| Pod length (cm) | 1.8 ± 0.07 | 1.8 ± 0.07 |
| Seed diameter (cm) | 0.90 ± 0.14 | 0.55 ± 0.07 |

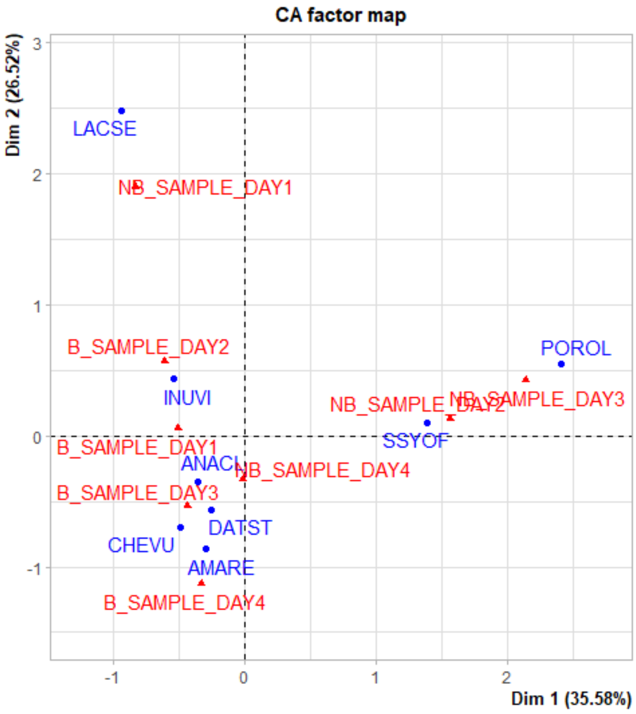
was not convenient for germination success and posterior plantlet development. This aspect will be studied in future research. It could be related to the amount of oxygen available. If there is too much moisture and the soil becomes compact, the seed could have less oxygen available to germinate adequately. When the biostimulant was sprayed on the soil after the sowing, the germination was promoted if the number of spray applications was 1-3; and on the contrary, it was inhibited if the number of spray applications was 5. Possibly, we reached a dose of biostimulant that resulted excessive for a proper germination. The biostimulant mixture used was positive for vegetative development in the flowerpot assay when applied in small quantities of liquid and was not positive in the field assay, probably because it also promoted the growth of weeds, which lessened the growth of the chickpea plants. In contrast, other research studies that inoculated chickpea seeds or soil with PGPB [2,3,20-24] obtained positive effects of biostimulants on chickpea plants. Chickpea seeds were inoculated with bacterial inoculum solutions several minutes before sowing and, consequently, seeds were not dry in the moment of sowing. It is necessary to develop new methods to apply the biostimulant on seeds which allows us to obtain dry seeds in order to be sown with a seed drill. If the grains are wet, the use of a conventional seeder is not possible so, we propose to apply the biostimulant 24h before sowing. Another option could be to apply the biostimulant sprayed on the soil just after the sowing and then, water to let it reach the seeds. The risk of this method is that biostimulant could promote, also, weed growth. When testing the implementation of a new biostimulant in chickpea, its application protocol is very relevant to its effect on the crop, according to our results. The seed previously wet and then let dry or exposed to a high amount of liquid could induce lower germination success, vegetative development and yield. The difference between the present research and other studies is that in this study we try to replicate the cultivation conditions of intensive agriculture under rainfed conditions. Chickpea grains should be treated with biostimulant in such a way that could be used in large extensions of land and under rainfed conditions. Because of this, we applied the biostimulant 24 h before sowing. In the field assay, it was applied directly to the seed 24 h before sowing using a small concrete mixer. This method is still being optimized and it is not discarded that such a way of application of the biostimulant could form a biopellicle around the seed that affects the germination, the exchange of nutrients at the beginning of the plantlet development or interfere with phytohormone action, plant biomolecules and/or nutrients. This could explain the results we obtained in two experiments about germination percentage, plant growth and phenological state. In future experiments, we are going to study different types of biostimulant and application protocols for chickpea crops. Another possible explanation of the negative effects of biostimulant on chickpea plants in the field assay is that it could have also promoted weeds, species better adapted to adverse conditions than chickpea plants. In the field assay, when studying the phenological stages of the plants, it was observed, from June 20, 2022, that there was a huge growth of *Amaranthus retroflexus* L. This lessened the growth of chickpea plants and displaced them to the edges of the land plot. In addition, another reason could be the different nutrients or seed banks in the land plots. Therefore, in the same way, that biostimulants promoted chickpea growth [2], they could promote weed growth, species more competitive than chickpeas. Correspondence analysis with the weeds showed that the distribution of weed species was different depending on the use or not of biostimulant on the land plots. We are going to continue experimenting with the protocol of biostimulant ap-plication and optimizing it to be of interest to intensive chickpea agriculture.

As chickpea roots are mycorrhized by *Rhizobium* sp., an atmospheric nitrogen-fixing microorganism, sometimes there are no differences in production between fertilized and unfertilized plots [4]. Because of this, maybe the focus of biostimulants is not the incorporation of N in the chickpea plants, but the activation of their defenses against diseases and pathogens to make the chickpea plants more competitive.

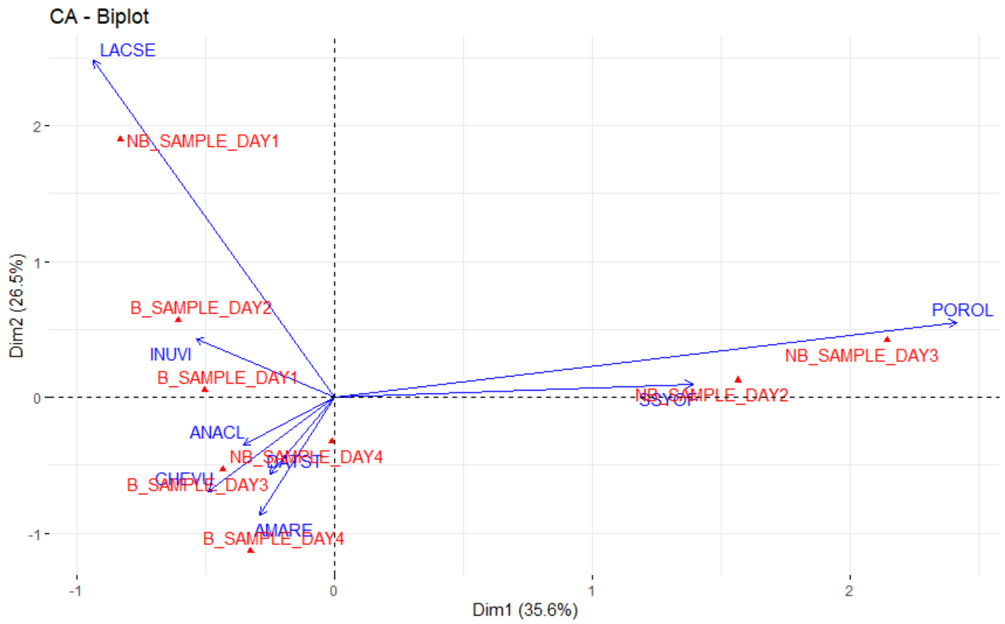
According to the correlation analysis of weeds, *Amaranthus retroflexus*, *Datura stramonium* and *Chenopodium vulvaria* are more correlated with later dates of observation and the presence of biostimulant. They are plants that need higher temperatures for their development. The biostimulant in chickpea seeds was also promoting these weed species and probably soil microfauna [25].

**Table 4. Weed species observed in the different land plots with Amelia chickpea variety without biostimulant (AM-) and with biostimulant (AM+)**

| Date | AM - | AM + |
|---|---|---|
| 05/05/2022 | *Dittrichia viscosa* (L.) Greuter<br>*Lactuca serriola* L. | *Dittrichia viscosa* (L.) Greuter<br>*Portulaca oleracea* L. |
| 27/05/2022 | | *Chenopodium vulvaria* L.<br>*Datura stramonium* L.<br>*Dittrichia viscosa* (L.) Greuter |
| 10/06/2022 | *Anacyclus clavatus* (Desf.) Pers.<br>*Datura stramonium* L.<br>*Dittrichia viscosa* (L.) Greuter<br>*Sisymbrium officinale* (L.) Scop. | *Amaranthus retroflexus* L. |




(**a**)




(**b**)

**Fig. 4. Correspondence analysis with the weeds observed in the photos of the land plots with Amelia without and with biostimulant: (a) CA factor map; (b) CA biplot: *Amaranthus retroflexus* (AMARE), *Anacyclus clavatus* (ANACL), *Chenopodium vulvaria* (CHEVU), *Datura stramonium* (DATST), Dittrichia viscosa (INUVI), *Lactuca serriola* (LACSE), *Portulaca oleracea* (POROL), *Sisymbrium officinale* (SSYOF)**

The phenological monitoring of chickpea plants revealed that on July 4, 2022 some pods, mainly in the superior parts of the plant, were empty. The reason for this fact is the high temperatures in the reproductive stage in May 2022, reaching temperatures over 30°C during 14 days in May, six of them consecutives, and drought, there was no rain except for May 21, 2022 and just only 0.2 mm. High temperatures induced early plant maturation. This could cause some pods to be empty and some seeds to present malformations and be smaller. There are a lot of references on how high temperatures during the reproductive stages of chickpea affect very negatively to pod formation and maturation yield. Temperatures over 30°C reduced grain number and weight and affected chickpea productivity [26-29]. Temperatures over 32°C aborted flowers and pods [30], leading to reduced seed size and yield [28,31-32]. Temperatures over 35°C in spring caused also flower abortion and a reduction in the time available for seed filling [33]. Heat-sensitive genotypes of chickpea have the synthesis of sugars decreased in the anthers due to enzyme inhibition. Plant pollen with lower sucrose levels is less functional, so fertilization is impaired and, pod and seed production poor [31-33]. High temperatures affect flowering, pollination and fruit development, causing a reduction in seed production and size, and yield [33-34]. According to GRDC [33], chickpea can tolerate high temperatures if there is adequate soil moisture.

The plants in the present field assay were also exposed to water stress because of the adverse climate conditions during May 2022. Drought affects various morphological and physiological processes of plants. Stress during the vegetative phase reduces plant size, leaf number and total leaf area, secondary branches, dry matter accumulation and the number of pods. Water deficits at the flowering and the post-flowering stages have been found to have greater impact than at the vegetative stage because it stops flowering and podding on the upper nodes and leads to early maturation. Irrigation at the stages of branching, flowering and pod formation improve yield [33,35].

Anticipation of the date of sowing (to January) avoided high temperatures and hydric stress during the reproductive stages of chickpea plants and its damaging effects on them and their yield (pers. com. of farmers). The search for chickpea varieties with earlier sowing dates and reproductive stages that do not coincide with drought periods is a possible adaptation to climate change [1].

## 5. CONCLUSION

Our results indicated that the way to apply the biostimulant on chickpea seeds was relevant with respect to germination success, vegetative development, and the phenological state of plants and it should be adapted to intensive agriculture with the use of a seed drill that requires dry seeds.

Regarding germination success, vegetative development, and phenological stages in the flower pot assay, the volume of the biostimulant mixture applied to the seeds was remarkable. Higher volumes of biostimulants affected negatively growth and development while lower volumes were positive.

The use of biostimulants was a negative influence, in the field assay, on chickpea vegetative development. It is probably due to the growth of weeds, which lessened the growth of chickpea plants.

Maybe the focus of biostimulants in chickpea cultivation is not fertilization, as in other crops, but the activation of plant defenses against pathogens to make chickpea plants more competitive.

The high temperatures and drought during the flowering stage in May 2022, reaching temperatures over 30ºC over many consecutive days, induced an early maturation of chickpeas plants and some pods were empty or dry, reducing the yield.

## ACKNOWLEDGEMENTS

The Organization responsible of this content is GO TecnoGAR. This research has been funded under the project GO-TecnoGAR, 80% co-financed by the EU (European Agricultural Fund for Rural Development, EAFRD) and 20% co-financed by the Spanish Ministry of Agriculture, Fisheries and Food. The Directorate General for Rural Development, Innovation and Agro-Food Training (DGDRIFA) is the management authority in charge of the application of the corresponding EAFRD and national aid. Total financing €432,329.05.
Jorge Hernández Sánchez is a recipient of a training fellowship funded by IMIDRA.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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