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Biostimulant derived from the fermentation of *Inula viscosa* (Inort) in the germination and growth of *Amaranthus hypochondriacus*

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Abstract

Research objective: This research aims to evaluate Inula's bio-stimulating potential in the form of macerate in water in the cultivation of *Amaranthus hypochondriacus*. This is to increase knowledge of this plant that commonly exhibits inhibitory characteristics towards the germination of various plants.

Materials and Methods: The experiments started in May 2022 and were conducted in the greenhouses of CREA-OF in Pescia on *Amaranthus hypochondriacus* plants. On September 24, 2022, plant height, leaves number, leaves the surface area, vegetative weight, roots volume and length, the number of germinated seeds, average germination time, the number of microorganisms in the substrate, plant dead number and pH were determined. In addition, the SPAD index was measured on three leaves pinched from the bottom to the apex of the canopy of each plant (for a total of 90 measurements per treatment) and tissue mineral content was evaluated on collected dry matter (N, P, K, Ca, Mg, Fe and Na, Cl).

Results and Discussion: The experiment showed that using Inula as a bio stimulant can significantly improve germination and growth and reduce mortality in *Amaranthus hypochondriacus* plants. In general, a significant increase in plant height and number of leaves, vegetative and root biomass was observed in plants treated with INORT, with differences depending on the percentage of product supplied to the plants. There was also a significant reduction in the mortality of plants treated with the biofertilizer product and increased microbial biomass. The trial also showed that the Inula biofertilizer could improve the chlorophyll content of the plant and increase the N, P, K, Ca, Fe, and Na content, while no differences were found for the parameters Mg and Cl.

This test also shows biostimulant effects regarding seed germination and the average germination time reduction. This is interesting because Inula is usually mentioned for its inhibiting activity in seed germination, which probably depends on the transformation process. However, when macerated with microbial products, the inhibiting activity is nullified, and the fast-growing microorganisms utilize the organic products provided by this plant.

Conclusions: In this experiment, further exciting and innovative aspects were highlighted in the use of this plant, given its already recognized importance from a medicinal, melliferous and biodiversity point of view. More experiments are underway to improve the protocol for using a biofertilizer based on Inula and microorganisms for horticulture for plant stimulation and defence.

Keywords: Amaranthus; Microorganisms; Sustainable agriculture; Biofertilizers; Rhizosphere

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1. Introduction

Amaranths are a wealthy family with about 70 varieties, many native to America. Highly decorative plants are often used in gardens; about twenty also have edible value. Already domesticated in prehistoric times as an agricultural plant, its original ancestor is unknown. There are now many variants of Amaranthus with very striking green to purple colours; these cultivars are divided according to their cultivation habitat and physical characteristics. Amaranthus follows the photoperiod flowering when it receives less than 15 hours of light per day [1, 2]. Most Amaranths do photosynthesis called C4, which is typical of many tropical and sub-tropical species such as maize, sugar cane, sorghum and euphorbia: this process is particularly efficient at high temperatures, in full sun and dry conditions; this makes it a helpful cultivar in harsh climatic conditions [3, 4]. A yellow-green dye is obtained from the whole plant. Amaranth is an annual plant that can grow up to 2 metres in height, flowering in the period from June to September. It is grown in full sun, in the ground or pots and is a self-seeding plant. The seeds and leaves are edible; the seeds are considered pseudo-grains suitable for gluten-free preparations [5]. They contain up to 16% protein; the calcium, phosphorus, magnesium and iron content is also remarkable. The whole plant contains tannins and is astringent; it is used internally to treat diarrhoea and excessive menstruation. In addition, it can be used to gargle in cases of mouth inflammation. Since ancient times, amaranth has been considered an emblem of immortality and a bearer of goodwill, as its flowers retain their colour and shape even when dry [6]. The Aztecs referred to it as the grain of the gods. It can be made into a red dye suitable for food, green or yellow for textiles. The variety sanguineus can be distinguished from hypochondriacus by the leaves' more significant red colouration [7].

1.1. Botanical description of *Inula Viscosa*

Inula Viscosa (syn. *Dittrichia viscosa* Greuter) (family Compositae) is a bushy, usually evergreen perennial, evergreen considered a weed, living in Mediterranean regions. It has a characteristic odour and is between 50 and 150 cm high. When touched, the leaves are linear-lanceolate and give off a characteristic pungent odour [8, 9, 10,11]. The terminal inflorescence is characterized by numerous flower heads with golden yellow flowers. Flowering in autumn, with fruits consisting of achenes. The plant is highly valued by research for the metabolites contained in the roots, stems, leaves and flowers, with medicinal properties also used for cosmetic purposes [12, 13, 14,15]. It treats numerous ailments such as liver problems, inflammations and infections. Several studies have been carried out to isolate the active compounds present in its biomass and identifiable in introductory classes of chemical compounds present in Inula are mainly the mono-, sesqui- and tri-terpenes, flavones, flavanones and carbohydrates [16, 17, 18,19]. Extracts of this plant have significant effects against *Candida albicans* fungal infections. The secondary metabolites of this plant are also known for their insecticidal, antifungal, acaricidal and antibacterial properties [20, 21]. In addition, several studies have highlighted the ability of *Inula Viscosa* in controlling the olive fly and in defending against varroa attacks in bees by an introduction in the form of a gel to create olfactory disorientation due to the pungent odour of the Inula released by the gel inside the hive [14].

1.2. Research Objectives

This research aims to evaluate Inula's bio-stimulating potential in the form of macerate in water in the cultivation of *Amaranthus hypochondriacus* (Figure 1). This is to increase knowledge of this plant that commonly exhibits inhibitory characteristics towards the germination of various plants. Previous work, however, has also revealed stimulating capabilities, using different maceration methods addicted with beneficial microorganisms that enhance the degradation of organic material and release valuable metabolites into solution. This trial, therefore, aims to increase knowledge of the stimulating activity of this plant on other plant species as well.

Figure 1 Detail greenhouse-grown *Amaranthus hypochondriacus* in the germination and complete growth phases

2. Material and methods

The experiments, which started in May 2022, were conducted in the greenhouses of CREA-OF in Pescia (Pt), Tuscany, Italy (43°54′N 10°41′E) on *Amaranthus hypochondriacus* (Figure 1A, 1B). The seeds were placed in ø 12 cm pots, 30 seeds per thesis, divided into three replicas of 10 seeds each. All plants were fertilized with a controlled release fertilizer (1 kg m^{-3} Osmocote Pro®, 9-12 months with 190 g/kg N, 39 g/kg P, 83 g/kg K) mixed with the growing medium before sowing. The experimental groups were:

- Group without Inula (CTRL) (peat 80%+ pumice 20%), irrigated with water and substrate previously fertilized;
- Group with Inula biofertilizer (1% every week) (INORT1) (peat 80% + pumice 20%) and fertilised substrate;
- Group with Inula biofertilizer (2% every week) (INORT2) (peat 80% + pumice 20%) and fertilized substrate.

The plants were watered one time a day and grown for six months. Then, the plants were irrigated with drip irrigation. The irrigation was activated by a timer whose program was adjusted weekly according to climatic conditions and the leaching fraction. On September 24, 2022, plant height, leaves number, leaves the surface area, vegetative weight, roots volume and length, the number of germinated seeds, average germination time, the number of microorganisms in the substrate, plant dead number and pH were determined. In addition, the SPAD index was measured on three leaves pinched from the bottom to the apex of the canopy of each plant (for a total of 90 measurements per treatment) and tissue mineral content was evaluated on collected dry matter (N, K, Ca, Mg, Fe and Na.

2.1. Analysis methods

- pH: For pH measurement, 1 kg of the substrate was taken from each plant, and 50 g of the mixture was placed in a beaker containing 100 ml of distilled water. After 2 hours, the water was filtered and analyzed [13];
- Microbial count: directly determining total microbial count by microscopy cells contained in a known sample volume using counting chambers (Thoma chamber). The surface of the slide is etched with a grid of squares, with the area of each square known. Determination of viable microbial load after serial decimal dilutions, spatula seeding (1 ml) and plate counting after incubation [13];
- Analytical instruments: IP67 PHmeter HI99 series - Hanna instruments; Combined test kit for soil analysis - HI3896 - Hanna instruments; Microbial diversity of culturable cells [13];
- AFTER DRY MATTER DIGESTION WITH SULPHURIC ACID, Reduced N was determined through Kjeldahl distillation. Dry matter was then subjected to nitric-perchloric acid digestion to determine: (i) P content through colourimetric method using a spectrophotometer; (ii) K, Ca, Mg, Fe and Na content through atomic absorption spectrophotometry [14].

2.2. Statistics

The experiment was carried out in a randomized complete block design. Collected data were analyzed by one-way ANOVA, using GLM univariate procedure, to assess significant ($P \leq 0.05$, 0.01 and 0.001) differences among treatments. Mean values were then separated by the LSD multiple-range tests ($P = 0.05$). Statistics and graphics were supported by the programs Costat (version 6.451) and Excel (Office 2010).

3. Results

The experiment showed that using Inula as a biostimulant can significantly improve germination and growth and reduce mortality in *Amaranthus hypochondriacus* plants. In general, a significant increase in plant height and number of leaves (Figure 2) and vegetative and root biomass was observed in plants treated with INORT, with differences depending on the percentage of product supplied to the plants (Table 1). The 2% INORT treatment was better than the 1% treatment. There was also an increase in leaf area 478.68 cm2 (INORT1) and 485.10 cm2 (INORT2) compared to the control with 457.53 cm2. The INORT treatment also elongated the roots; INORT1 and INORT2 showed an average elongation of 1-2 cm more than the control (Figure 3). There was also a significant reduction in the mortality of plants treated with biofertilizer. Another effect was the increase in microbial biomass in the theses treated with INORT (Table 2); the microorganisms probably use the metabolites contained in this plant as a nutrient substrate for their multiplication.

In contrast, only INORT2 showed a slight significant change in the pH of the cultivation substrate. The trial also showed that the Inula biofertilizer could improve the chlorophyll content of the plant; the theses treated with INORT showed a significant increase in this parameter compared to the control, regardless of the treatment dose. Finally, regarding the

mineral content of the plant tissues of *Amaranthus hypochondriacus*, the INORT-treated theses showed a significant improvement in the N, P, K, Ca, Fe, and Na content, while no differences were found for the parameters Mg and Cl (Table 3).

Table 1 Evaluation of the use of Inula-based biofertilizer on the vegetative and root biomass of *Amaranthus hypochondriacus*

Groups	Plant height (cm)	Leaves number (n°)	Leaves surface area (cm2)	Vegetative Weight (g)	Roots Volume (cm3)	Roots Length (cm)
CT	15.36 c	4.80 c	457.53 c	45.20 c	20.88 c	12.93 c
INORT1	16.71 b	6.20 b	478.68 b	47.66 b	23.36 b	13.31 b
INORT2	17.21 a	8.00 a	485.10 a	49.68 a	24.71 a	14.06 a
ANOVA	***	***	***	***	***	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05).Legend: (CT) control; (INORT1) Inula 1% ;(INORT2) Inula 2%

Table 2 Evaluation of the use of Inula-based biofertilizer on seed germination and microbial biomass of *Amaranthus hypochondriacus*

Groups	Germinated seed (n°)	Average germination time (days)	Substrate total bacteria (Log CFU/g soil)	pH substrate	Plants dead number (n°)
CT	21.00 b	14.00 a	5.45 b	6.83 b	1.20 a
INORT1	24.40 a	13.41 ab	5.93 a	6.84 b	0.20 b
INORT2	25.20 a	12.82 b	6.05 a	6.90 a	0.20 b
ANOVA	***	*	***	***	**

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05); Legend: (CT) control; (INORT1) Inula 1% ;(INORT2) Inula 2%

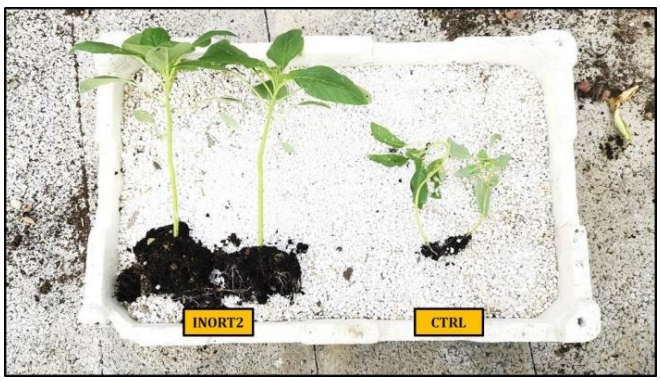

Figure 2 Comparison of Inula macerated at 2% (INORT2) and fertilized control on the vegetative growth of *Amaranthus hypochodriacus*

Table 3 Spad analysis and Mineral content of vegetative tissues of *Amaranthus hypochondriacus*

Groups	Spad	N (g/kg)	P(g/kg)	K(g/kg)	Ca(g/kg)	Mg(g/kg)	Fe(g/kg)	Na(g/kg)	Cl(g/kg)
CT	29.60 b	22.45 c	2.42 c	18.73 c	28.35 c	6.11 a	0.18 c	7.61 b	15.29 b
INORT1	35.44 a	23.70 b	2.70 b	19.63 a	29.16 b	6.10 a	0.27 b	7.80 a	15.65 a
INORT2	35.61 a	23.88 a	2.91 a	19.11 b	29.73 a	6.18 a	0.32 a	7.70 ab	15.27 b
ANOVA	***	***	***	***	***	ns	***	**	ns

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CT) control; (INORT1) Inula 1% ;(INORT2) Inula 2%

Figure 3 Effect of 2% Inula macerate (INORT2) on root growth of *Amaranthus hypochondriacus*

4. Discussion

The use of microbial macerate of *Inula Viscosa* has fertilizing properties and effects in controlling plant mortality, improving seed germination and increasing the vegetative and root development of *Amaranthus hypochodriacus* plants, as well as improving the mineral content of tissues. Indeed, as shown in previous trials, the mineral and metabolite content in *Inula Viscosa* can positively influence soil microbiology, which interacts with the plant by improving development, tissue nutrient content, increasing yield and reducing plant mortality.

The GC-MS analysis performed in previous trials of the active fractions strongly suggested costic acid as the compound responsible for controlling fungal and bacterial pathogens—an acid used in the literature to control *varroa destructor* [13]. In Inula-treated plants, resistance induction mechanisms of the plants certainly occur, which ensure a higher resistance to stresses of a biotic and abiotic nature. Previous trials have emphasized the ability of the secondary metabolites present in this plant for their insecticidal, antifungal, acaricidal, antibacterial and cytotoxic activity [22, 23, 24, 25]. This test also shows biostimulant effects regarding seed germination and the average germination time reduction. This is interesting because Inula is usually mentioned for its inhibiting activity in seed germination, which probably depends on the transformation process. When macerated with microbial products, the inhibiting activity of Inula is nullified, and the fast-growing microorganisms utilize the organic products provided by this plant. It is, therefore, attractive how the biostimulant or inhibiting activity of a plant can be modulated according to the transformation process. This plant is also essential because it contributes to the production in late summer and autumn of multi-flower honey and, in areas with a strong diffusion, monofloral honey. The *Inula Viscosa* is attacked by *Myopites stylus*, a gallsitter Tephritid [12, 13]. This insect is the overwintering host of *Eupelmus urozonus*, a polyphagous parasitoid of *Hymenoptera Calcididae*, which carries out 2-3 generations per year on the olive fly. Since Eupelmus is the most active natural antagonist of the olive fly, the spread of the Inula in uncultivated areas of olive groves is essential because it can contribute to olive fly control [14].

5. Conclusion

The test showed that using a liquid biostimulant based on *Inula Viscosa* can significantly improve the seed germination, plant growth and the quality of *Amaranthus hypochondriacus*. Furthermore, the continuous application of this biostimulant also reduced plant mortality. Furthermore, it improved the nutritional profile of vegetative tissues, an aspect probably influenced by the increased microbial biomass in the substrate. Therefore, in this experiment, further

exciting and innovative aspects were highlighted in the use of this plant, given its already recognized importance from a medicinal, melliferous and biodiversity point of view. More experiments are underway to improve the protocol for using a biofertilizer based on Inula and microorganisms for horticulture for plant stimulation and defence.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declares no conflict of interest.

Statement of ethical approval

The present research work does not contain any studies performed on animal/humans subjects.

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