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Long-Term Field Evaluation of Conventional vs. Micropropagated Plants of *Chrysanthemum cinerariifolium*

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Abstract: Pyrethrum is a perennial herbaceous plant endemic to the eastern coast of the Adriatic Sea, and introduced in large areas of nearly all continents, where it is cultivated for the industrial extraction of pyrethrins. Pyrethrins are a group of six closely related monoterpene esters, widely used as natural insecticides. The world production of natural pyrethrins is lower than the market demand, and a wider introduction of this crop within the Mediterranean agrosystems could be an appealing opportunity for farmers and manufacturers. The availability of adequate amounts of selected plant material to bring into cultivation is, however, one of the major issues. Therefore, the in vitro propagation of elite pyrethrum genotypes could be a suitable alternative to conventional propagation methods. In this paper, we present the results of a 9-year field comparison between pyrethrum plants coming from an in vitro propagation protocol and plants obtained by cutting from the same mother plants. Furthermore, since plantlets derived from in vitro regeneration may experience ploidy changes, we evaluated the stability of the ploidy level of pyrethrum-micropropagated plants by flow cytometry (FCM) analysis. FCM screening revealed no differences among the morphotypes and between them and the mother plant. Likewise, the field evaluation of plants gave no significant differences between flower yields in both groups. Hence, micropropagation was confirmed as an easy, efficient and reproducible method to obtain large quantities of selected pyrethrum genotypes.

Keywords: establishment in field; ex vitro acclimatization; flow cytometry; micropropagation; natural insecticides

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1. Introduction

Chrysanthemum cinerariifolium (Trevis.) Vis. = *Tanacetum cinerariifolium* (Trevis.) Sch.Bip. *(Asteraceae)*, commonly known as pyrethrum, is endemic to the Eastern coast of the Adriatic Sea [1]. It is an important perennial industrial crop, grown worldwide for the production of pyrethrins, a group of six closely related monoterpene esters with insecticidal properties [2–4].

Pyrethrins' content is influenced by several factors, such as the development stage of flower heads [5,6], morphological traits (above all, floral characteristics: flower size, floral weight, number of flowers, flower yield, etc.), genotype, climatic and edaphic factors and agronomic practices [7].

The pyrethrum flower head is composed of white female ray florets on the margin and yellow bisexual disk florets in the center of the receptacle [8,9]; both the ray and the disc flowers form the fruits (achenes). Pyrethrins are mainly stored inside small oil glands located on the external surface of flower head achenes (about 94%) [6,10].

Thanks to its low toxicity to mammals, other warm-blooded animals and the environment, pyrethrum is the only plant species whose metabolites are currently traded as insecticides.

The world production of natural pyrethrins is lower than the market demand, and a wider introduction of this crop within the Mediterranean agrosystems could be an appealing opportunity for farmers and manufacturers. The availability of adequate amounts of selected plant material to bring into cultivation is, however, one of the major issues, and the in vitro propagation of pyrethrum could be a valid alternative to conventional propagation methods.

The first large-scale application of in vitro tissue cultures for the mass propagation of pyrethrum clones with superior production characteristics was conducted in Ecuador [11], where, in the conventional propagation pathway, some issues were reported, mainly related to the maintenance of production characteristics. For this reason, in vitro culture was used as an alternative for the mass propagation of selected clones of pyrethrum; considerable advantages have been found in this application compared to conventional propagation procedures, such as savings in terms of time, improvement in plant growth characteristics (greater vigor, better stress resistance) and independence from climatic and geographical conditions. However, other questions are still to be solved. First, pyrethrum, as a perennial species, has complex seasonal and life cycles, which complicate the control of plantlets' growth in microculture [12]. Second, it cannot be excluded that plantlets derived from in vitro culture may exhibit ploidy variations. In vitro cultures are prone to mutations induced by several factors. Explant source and type, age of culture and number of subculture cycles, and plant growth regulators used in propagation and regeneration trials influence the onset of somaclonal variability [13,14]. Therefore, it is necessary to evaluate the genetic stability of micropropagated plants to ensure their compliance with mother plants. Flow cytometry (FCM), a reproducible technique for estimation of the nuclear genome size in plants, is a reliable evaluation system for young plantlets obtained by in vitro micropropagation [15].

In this study we compared the field performance of pyrethrum plants micropropagated in vitro with plants obtained by conventional cuttings after verifying ploidy stability by FCM analysis.

2. Materials and Methods

2.1. Plant Material

The experimental activity on pyrethrum started in 2008 when a core collection of plants (10 specimens) obtained from the same commercial source was established in the experimental farm "Sparacia" (Cammarata, AG—Sicily; 37°38′ N–13°46′ E; 415 m s.l.m.). Soil was a vertic xerofluvent [16], with a clayey texture, and a poor content of nitrogen and organic matter. The plants were transplanted at spacings of 60 × 60 cm, on pockets 30–40 cm deep, each previously fertilized with about 5 kg of ripe bovine manure. In the following years, the plot was manually kept free from weeds; no other fertilizers were supplied, and irrigations were performed in the summer months according to plants' necessity. In summer 2010, mature achenes were collected from flowered plants and used for axenic culture experiments.

2.2. In Vitro Culture and Acclimatization

Micropropagated plants were obtained in vitro as previously described ([6], with minor modifications), using young plantlets issued from seeds (Figure 1).

To stimulate germination, seeds were treated with 1 mg/mL Gibberellic acid (GA_3), and submitted to prechilling at 4 °C for 8 days. After that, seeds were surface disinfected under laminar flow by immersion for 10 min in commercial 20% bleaching solution (with 2.2% active chlorine), followed by three 5 min rinses with sterile distilled water.

Seeds were cultured on Murashige and Skoog medium (MS) [17], (Duchefa Biochemie, Haarlem, The Netherlands), with 30 g/L of sucrose as carbon source and 8 g/l of Plantagar; the pH was adjusted to 5.6 ± 0.1 with 1 M KOH before autoclaving at 121 °C and 1 atm for 20 min. For germination tests, we used Petri dishes 10 cm in diameter by 2.5 cm in height and 25 mL capacity, sealed with Parafilm M^{TM}. Petri dishes, each containing 15 seeds, were

incubated in a growth chamber at 15 ± 1 °C under a 16 h day length, and a photosynthetic photon flux of 50 µmol m^{-2} s^{-1} provided by Osram cool-white 18 W fluorescent lamps.

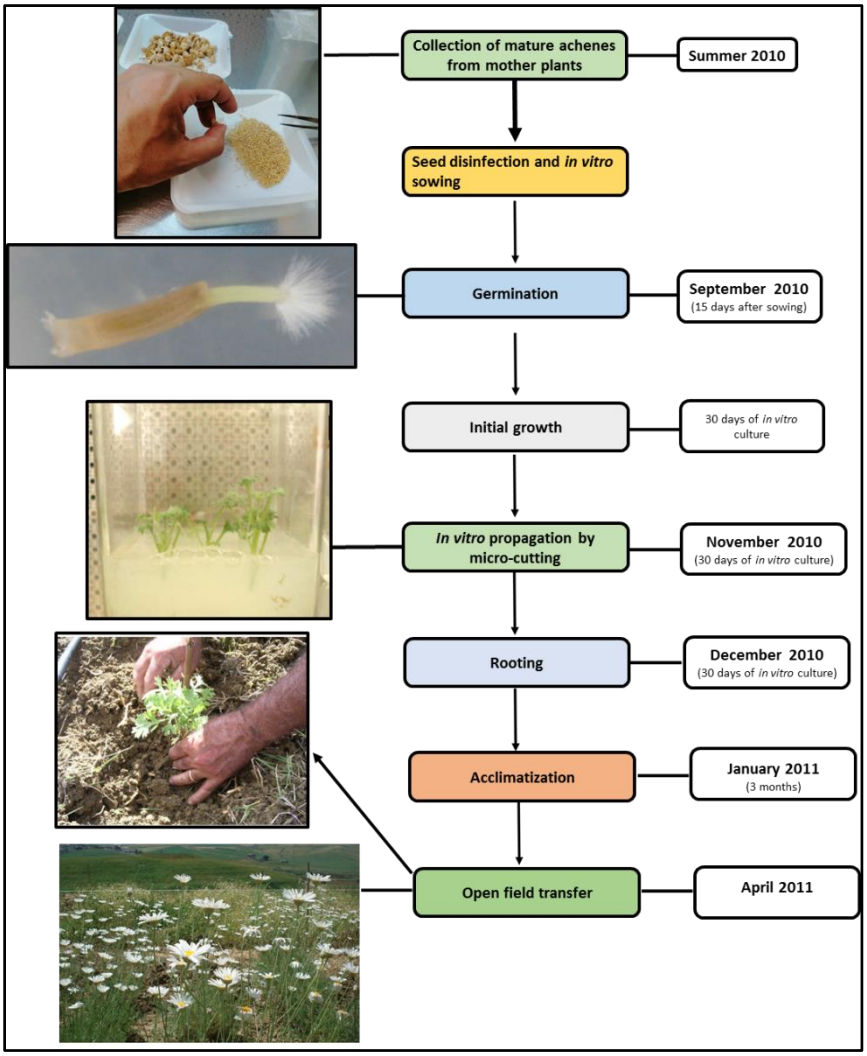

Figure 1. In vitro propagation of pyrethrum: flow chart.

At the end of the 15th day, germination percentage was calculated. Subsequently, in order to obtain enough plant material for rooting step, plantlets were transferred in sterile Magenta boxes containing 60 mL MS medium with no plant growth regulators and subcultured at 30-day intervals. Green shoots (2–3 cm long) were collected after 30 days and used for in vitro rooting.

Rooted shoots were collected 30 days after rooting treatments and washed with tap water in order to remove the medium before being transplanted individually into plastic pots 70 mm × 70 mm containing sterile soil. To maintain proper levels of temperature and humidity, the potted plants were covered with bags in transparent polyethylene, then placed in a climate chamber at 25 ± 1 °C under the previously described illumination conditions. After 20–30 days, plantlets were gradually exposed to reduced humidity, and after 40 days they were transferred outdoor under natural daylight conditions. A shadowing net was used to allow the final acclimatization.

The survival rate was recorded after a three-month acclimatization period, when plantlets reached an average height of 15–20 cm with 5–6 expanded leaves and functional roots. In April 2011, they were transferred in open field.

2.3. Assessment of Ploidy through Flow Cytometry

FCM was used to analyze the relative nuclear DNA content of leaf cells collected from the in vitro micropropagated plants. Each sample was analyzed with an internal diploid standard made of the respective mother plant's leaf nuclei (STD 2C).

The analysis was carried out using the Partec PAS flow cytometer (Sysmex Partec, Görlitz, Germany, https://www.sysmex-partec.com/ (accessed on 26 May 2022), equipped with a mercury lamp. Nuclei were released from cells obtained from 0.5 cm^3 of leaves collected both from micropropagated and mother plants by chopping in 300 µL nuclei extraction buffer (solution A of the "High Resolution Kit" for PlantDNA, Sysmex Partec, Görlitz, Germany) for 1 min. The suspension of nuclei was filtered through a 30 µm Cell-Trics disposable filter (Cell-Trics Sysmex Partec, Görlitz, Germany), 1.2 mL staining solution containing the dye 4,6-diamidino-2-phenylindole (DAPI; solution B of the kit) was added. DNA Control UV (Sismex Partec—Görlitz, Germany) was used for alignment of the instrument and setting up and validating the device for high analysis resolution and signal linearity.

For each sample of micropropagated plants and internal diploid standard, 3 replicates were performed.

Using the Partec FlowMax software package Version 2.3, 3000–4000 nuclei were measured per sample and histograms of DNA content were generated.

2.4. Conventional Cuttings

In summer 2010, vegetative shoots (about 10 cm long, and with 4–5 developed leaves) were excised from the basal part of mother plants and transplanted for rooting in plastic pots filled with a 1:1 sand–perlite mixed substrate. In April 2011 (the same period of transplant of micropropagated plants), mature plants obtained in vivo from cuttings were transplanted in open field.

2.5. Open Field Management and Calculation of Thermal Sums

Conventional and micropropagated plants were arranged in field on a soil similar to the soil used for growing the mother plants. Two separated plots were therefore obtained, containing 20 plants each, one with plants obtained by cuttings, and the other plot with in vitro micropropagated plants. Plants within each plot were spaced at 50 × 60 cm each (60 cm between rows, 50 cm between plants on row). In both plots, the plants were periodically surveyed each year until harvest time, and their phytosanitary state and general development conditions were monitored. No treatment against pests was necessary; only weeds were controlled manually in spring once a year, shortly before the beginning of the reproductive stage, identified with the emission of flower buds. Fertilization consisted of a ripe bovine manure supply, distributed only once in 2011, before transplant, as previously applied to mother plants. Plants were watered only in spring and summer in the first year, throughout the whole cycle of plant from the restarting of vegetation to the full flowering, by distributing a water supply based on visual assessment of plants' conditions. In all subsequent years, no additional watering was performed, and plants relied, for their growth and production, only on natural rainfall events.

The trends of rainfall and temperatures recorded in Sparacia throughout the whole trial period are reported in Figure 2. Temperature daily measurements of all years were used to calculate the thermal sums (°C) until the appearance of flower buds and full flowering (harvest time), using the following formula

$$TS = \sum_{i=1}^{k} \left(T_{avg} - T_{base}\right)_I$$

where: i and k: first and last days of measurement, respectively; T_{avg}: daily average temperature; T_{base}: base temperature. When $T_{avg} < T_{base}$, the daily thermal sum was zero.

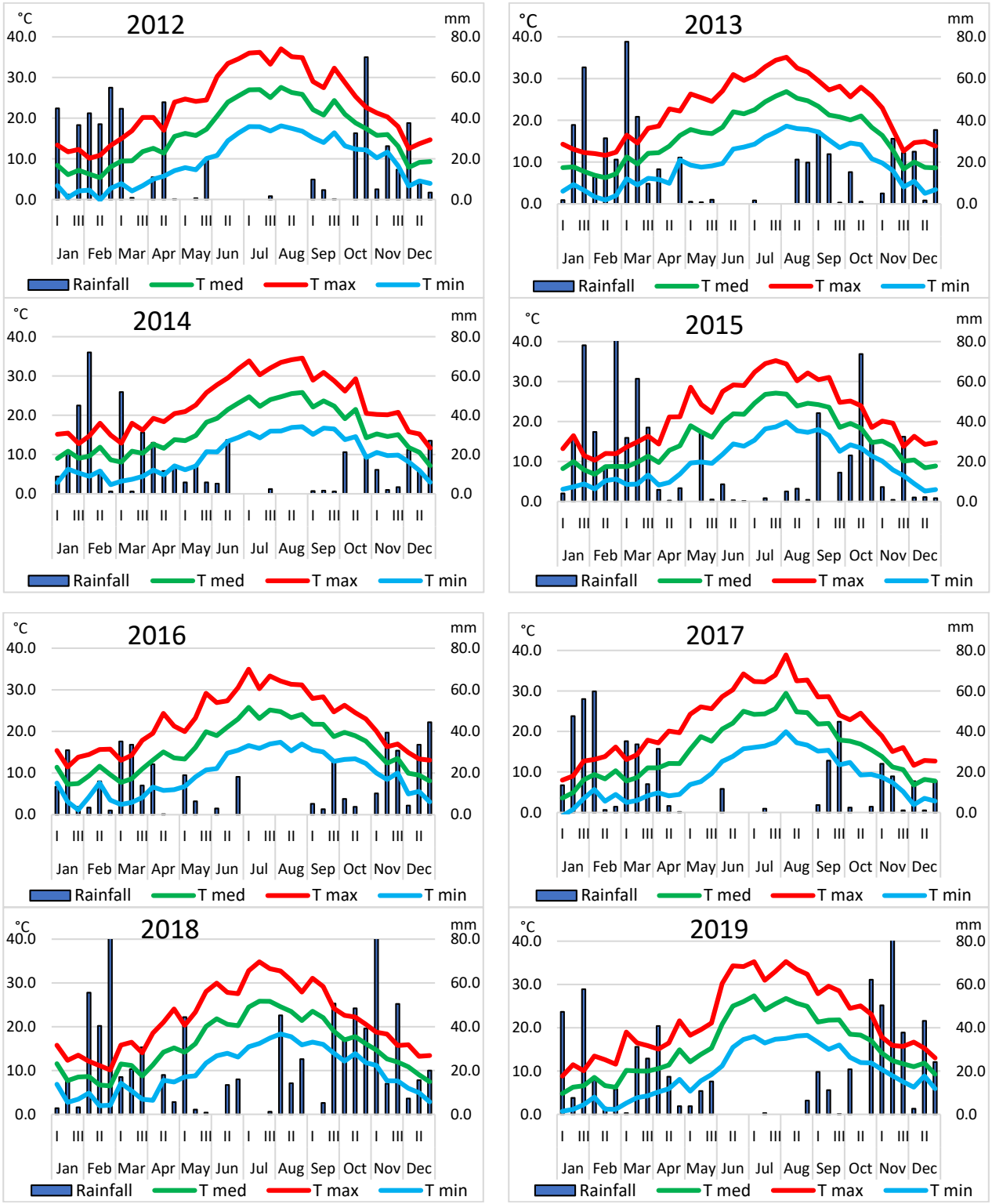

Figure 2. Ten-day values of rainfall (mm) and temperatures (minimum, maximum and mean; °C) recorded at the "Sparacia" farm (Cammarata, AG, Italy) from 2012 to 2019.

Calculations were assumed to start on the 1st day of January of each year (day i) and to conclude at the appearance of flower buds (FB) and at full flowering (F; harvest time). T_{base}, i.e., the temperature value below which plant growth is assumed to be zero, was set equal to 10 °C [18].

From 2012 to 2019, plants were harvested each year at full flowering time, which occurred between late May and early June, without any apparent difference between conventional and micropropagated plants. To allow a quick regrowth in the following year, all plants were cut at ground level, and random samples of five plants per treatment were taken, and their weight (g), height (cm) and number of stems per plant were measured. The height of the first flower was measured in field before harvest.

2.6. Statistical Treatment of Data

All data were submitted to variance analysis (ANOVA), by means of the statistical package Minitab® version 17.1.0 (Minitab Inc., State College, PA, USA, 2013). Prior to analysis, variance homogeneity was checked in all the investigated variables by means of the Levene's test, implemented in the same software. A General Linear Model (GLM) procedure was used, setting as dependent variables all the measurements obtained in the trial, and as independent variables the factors "year" (treated as a random factor) and "propagation method" (treated as a fixed factor).

When the ANOVA gave a significant response, the differences between mean values were evaluated through Tukey's post hoc comparison test, also implemented in the Minitab package.

Finally, to identify any significant relation between the obtained plants' traits and the measured environmental parameters, Pearson's "*r*" simple correlation coefficients were calculated between each measurement on plants (including both conventional and micropropagated plants), the thermal sums accumulated until appearance of flower buds and flowering time, and the amount of rainfall measured in the same stages. Correlation coefficients were calculated by means of the PAST statistical package version 4.04 [19,20].

3. Results

3.1. In Vitro Culture and Acclimatization

In vitro sowing provided several seedlings for the micropropagation step. As previously described, shoots exhibited a satisfactory growth rate and root induction. The percentages of rooted plants reached 100% 8–10 days after culture initiation. The acclimatization of the in vitro raised plantlets reached 60% success; thereafter, they were transferred in open field conditions.

3.2. Analysis of Ploidy Level

FCM analysis was performed to verify potential differences in the ploidy level of in vitro plants after micropropagation.

The presence of a single peak in all analyzed samples at channel 200 indicates the accumulation of homogeneous 2C nuclei (Figure 3), with no differences in ploidy between the plants studied. Furthermore, the in vitro micropropagated plants had the same ploidy as the mother plant, indicating that polyploidization did not occur.

3.3. Field Evaluation

The variance analysis (Table 1) showed that, in all tested variables, the interaction "year" × "propagation method" was always not significant. Hence, the studied plant traits were independently affected by the year and by the propagation method. The propagation method gave significant differences only in the height of plants, the weight of plants and the height of the first flower. The data from Table 1 show that micropropagated plants reached, on average, height values around 56 cm, a one-plant weight approaching 135 g, and about 32 ramifications/plant. Plants obtained by conventional cuttings were significantly taller (61.4 cm) and heavier (181 g), although this different plant size did not bring any significant variation in flower number (74–87 flowers/plant) and yield (40–51 g/plant). The height of the first flower is an agronomically relevant character, since a high, uniform and compact flowered layer is related to the mechanizability of the crop; probably due to their overall

bigger size, conventionally obtained plants exhibited a higher value of this trait (about 39 cm vs. 35 cm in the micropropagated plants).

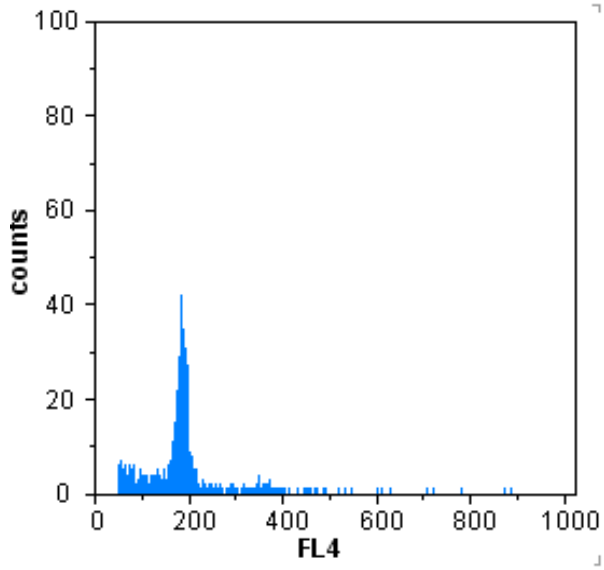

Figure 3. Representative flow cytometric histograms. Relative fluorescence intensity (FL4) obtained after simultaneous analysis of nuclei isolated from leaves of pyrethrum mother plant and micropropagated plants.

The major plants' traits observed during the trial exhibited a strong variability in years. A definite increasing trend over years was evident in plant height, plant weight, the number of stems per plant, and the number and weight of flowers per plant from 2012 to 2014, when the highest values were recorded for all these parameters.

Plant height varied between 39.5 cm, recorded in 2017 (the least favorable year), to 68.5 cm in 2014.

Plant diameter expressed a similar variation, ranging from 32.5 cm in 2013 to 47.9 cm in 2014.

Plant weight (g) ranged from 61.2 g in 2017 to 293.5 g in 2014; the number of stems per plant reached its lowest value in the first year (10.2 stems/plant) and the highest in 2019 (64.1 stems/plant), and the height of the first flower showed its lowest value in 2017 and the highest in 2015 (46.1 cm).

Both traits expressing a plant's productivity, i.e., the number of flowers per plant and the yield of flowers per plant, had the lowest values in 2012 (50.3 flowers/plant and 18.9 g, respectively) and the highest in 2014 (153.4 flowers/plant and 96.8 g, respectively).

The correlation analysis (Figure 4) shows some relationship between the observed traits, as well as between those traits and the calculated climatic parameters. Measurements of the plants' size (height, weight, diameter and number of ramifications) were always intercorrelated, and those concerning flower yield (number and weight of flowers per plant) showed a significant correlation with plant diameter, plant weight and number of stems per plant. The yield of flowers was highly correlated with their number per plant ($r = 0.911$, $p < 0.01$). Significant negative correlations showed up between flower yields measured in all 9 trial years and the respective durations of the stages preceding flowering time, above all, those before the appearance of flower buds. The duration of each substage (until the emission of flower buds and between this moment to full flowering time), was directly correlated to heat accumulation, but, since only low heat accumulation was possible in winter and early spring, this association was significant ($r = 0.746$, $p = 0.03$) only between thermal sums and the number of days that elapsed from the appearance of flower buds to the full flowering stage.

Table 1. Pyrethrum (*Chrysanthemum cinerariifolium*). Mean values ± SE and results of the ANOVA for the major morphological and yield characteristics recorded at Sparacia (Cammarata-AG) from 2012 to 2019.

Source	DF	Plant Height (cm)		Plant Diameter (cm)		Plant Weight (g)		N. of Stems/Plant		Height 1st Flower (cm)		N. Flowers/Plant		Yield of Flowers/Plant (g)	
Year (Y)	7														
2012		61.13 ± 2.1	AB	36.46 ± 2.7	BC	116.14 ± 4.0	CD	10.17 ± 1.1	D	40.37 ± 1.9	AB	50.30 ± 6.6	B	18.93 ± 2.7	B
2013		62.08 ± 2.6	AB	32.49 ± 1.3	C	123.19 ± 5.4	CD	31.62 ± 3.7	BC	38.95 ± 1.6	AB	90.50 ± 11.1	AB	34.91 ± 4.1	B
2014		68.52 ± 2.1	A	47.86 ± 4.2	A	293.5 ± 23.5	A	54.63 ± 5.2	A	31.32 ± 2.6	B	153.4 ± 18.8	A	96.80 ± 14.2	A
2015		65.35 ± 1.9	A	43.55 ± 1.5	AC	199.67 ± 9.2	B	30.41 ± 4.3	BC	46.10 ± 1.5	A	78.80 ± 10.3	B	55.84 ± 7.5	AB
2016		60.10 ± 3.2	AB	41.72 ± 2.1	AC	178.7 ± 11.1	BC	30.24 ± 2.4	BC	42.18 ± 2.2	AB	68.96 ± 9.9	B	48.19 ± 3.3	B
2017		39.47 ± 1.9	C	35.39 ± 2.9	BC	61.16 ± 8.1	D	24.33 ± 2.8	CD	17.63 ± 1.8	C	60.33 ± 9.2	B	24.63 ± 3.3	B
2018		50.50 ± 4.9	BC	42.48 ± 2.0	AC	131.9 ± 14.5	BD	46.29 ± 6.8	AB	38.32 ± 3.7	AB	85.30 ± 13.6	AB	41.95 ± 4.0	B
2019		57.84 ± 2.2	AB	43.64 ± 0.9	AC	162.49 ± 8.9	BC	64.05 ± 2.9	A	41.44 ± 2.2	AB	87.20 ± 10.3	AB	55.10 ± 1.9	AB
F-value $_{(7, 52)}$		11.08 ***		4.85 ***		29.99 ***		18.71 ***		15.35 ***		7.28 ***		12.55 ***	
Error$_a$	52														
Propagation method (PM)	1														
Cuttings		61.40 ± 1.8	A	39.20 ± 1.3		181.1 ± 12.4	A	34.12 ± 2.5		38.99 ± 1.7	A	88.69 ± 6.1		51.16 ± 3.9	
In vitro micropropagated		56.03 ± 1.7	B	41.20 ± 1.5		134.86 ± 7.7	B	32.07 ± 3.3		35.40 ± 1.6	B	74.30 ± 8.0		40.35 ± 5.3	
F-value $_{(1, 24)}$		8.10 **		2.11 n.s.		5.05 *		1.40 n.s.		10.82 **		<1 n.s.		<1 n.s.	
PM *Y	7														
F-value $_{(7, 24)}$		2.30 n.s.		<1 n.s.		<1 n.s.		1.30 n.s.		2.33 n.s.		1.54 n.s.		<1 n.s.	
Error$_b$	24														

*, **, ***: statistically significant values, at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively; n.s.: not significant. For each character and source of variability, means not followed by the same letters (including not reported intermediates) are significantly different at $p \leq 0.05$ (Tukey's test).

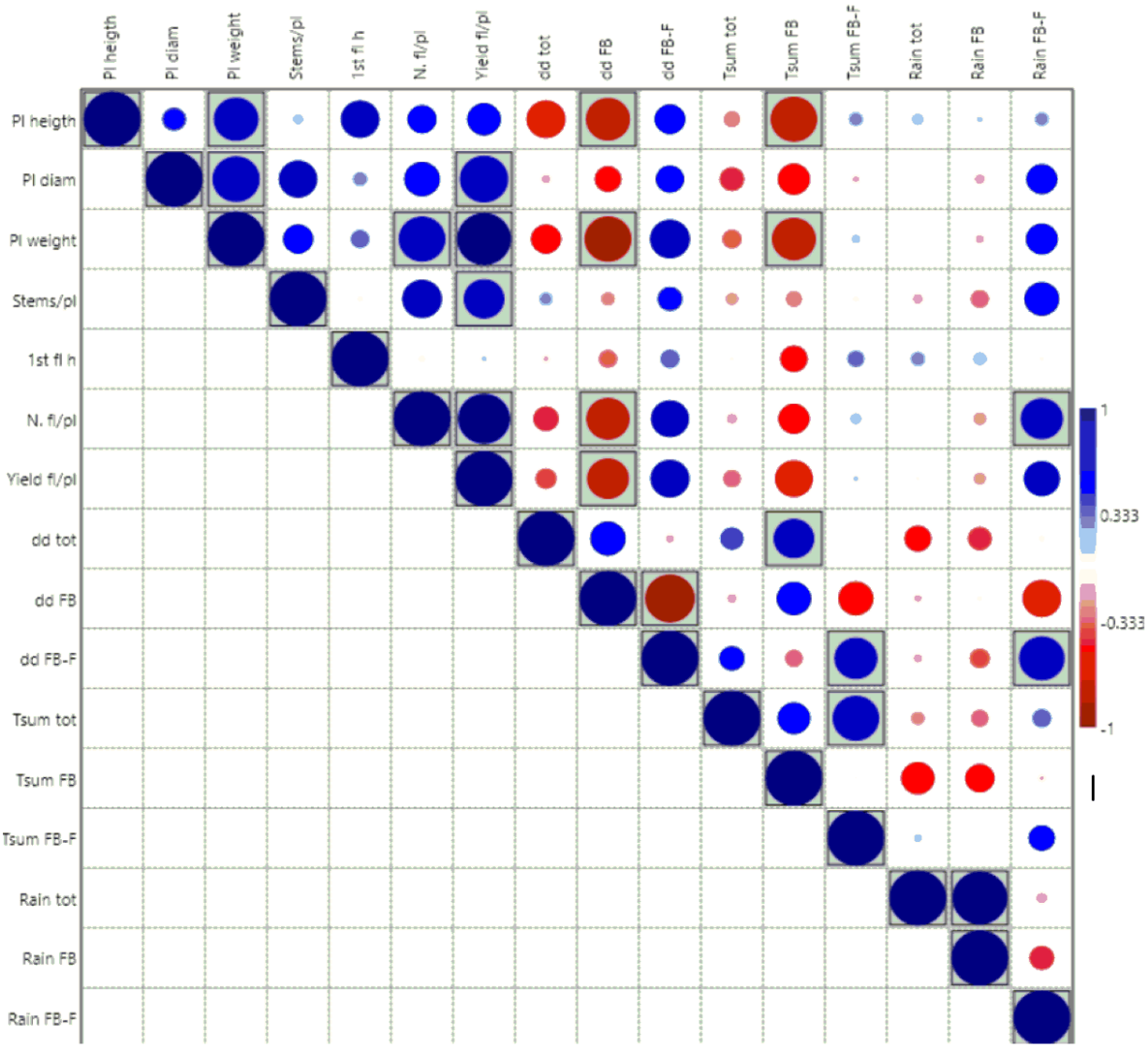

Figure 4. Correlations (Pearson's *r* values) plot between the major traits measured at Sparacia (Cammarata, AG, Italy) on pyrethrum plants from 2012 to 2019 and the correspondent climatic parameters. Legenda: Pl height: plant height (cm); Pl diam: plant diameter (cm); Pl weight: weight of 1 plant (g); Stems/pl: n. of stems/plant; 1st fl h: height of the 1st flower (cm); N. fl/pl: n. of flowers/plant; dd tot: n. of days from 1 Jan to full flowering; dd FB: n. of days from 1 Jan to appearance of flower buds; dd FB-B: n. days from appearance of flower buds to full flowering; Tsum tot: thermal sum from 1 Jan to full flowering (°C); Tsum FB: thermal sum from 1 Jan to appearance of flower buds (°C); Tsum FB-B: thermal sum from appearance of flower buds to full flowering (°C); Rain tot: rainfall amount from 1 Jan to full flowering (mm); Rain FB: rainfall amount from 1 Jan to appearance of flower buds (mm); Rain FB-B: rainfall amount from appearance of flower buds to full flowering (mm). Boxed r values are significant at $p < 0.05$.

Rainfall amount recorded during plant growth (Figure 2), spanning from 202 mm (in 2016) to 458 mm (in 2015), could always fulfill plants' requirements; hence, since no constraining condition was recorded during the trial, the correlation analysis did not detect any significant association between these values and yields. The significant association detected between the number of flowers/plant and the rainfall amount recorded after the bud emission stage (r = 0.735, $p = 0.04$), however, allows the argument that this additional rainfall could allow more flowers to open, indirectly enhancing flower yield.

4. Discussion

Micropropagation is an effective method for large-scale plant production, offering a number of benefits. First of all, regardless of the season, the time period required for the in vitro plant propagation is generally much shorter than the time needed for conventional propagated plants.

Furthermore, in vitro propagation allows the multiplication of a huge number of propagules in a very limited space. Moreover, by using tissue culture, plants are supposed to enhance their production of bioactive secondary metabolites [21].

Compared to traditional stem cutting propagation, micropropagation also requires the maintenance of fewer mother plants [22].

The use of in vitro grown explants is advantageous as it reduces the difficulty relating to culture contamination [23,24].

However, one of the main problems facing the in vitro culture is the occurrence of somaclonal variations in plants [14,25] that could be the result of changes in the ploidy level in response to the in vitro culture conditions [26,27]. Particularly when plants are considered as a source of metabolites with biological activity, it is crucial to confirm their ploidy level since the chemical composition of these secondary metabolites should remain unchanged after micropropagation [28].

Therefore, it is necessary to evaluate the ploidy stability of micropropagated plants to ensure their compliance with mother plants. Ploidy variation in micropropagated plants can be detected by flow cytometry, although this method could mask the possible occurrence of small differences in the content of nuclear DNA (aneuploidy and DNA polymorphism), and, therefore, complementary studies to assess this situation, such as chromosome counts and microsatellites, must be carried out [29,30]. Flow cytometric analysis showed no ploidy level variation among in vitro micropropagated plants.

The reported data allow some of the mechanisms underlying flower yield in pyrethrum to be shown. In general, the emission of flowers was dependent on plant size, meaning that bigger plants can bear a higher number of ramifications and flowers. The size of plants (expressed by height and weight of plants) was, however, inversely associated with the duration of vegetative stages, above all, those preceding the emission of flower buds, meaning that a higher heat accumulation, as caused by a longer period before the reproductive phase, was detrimental to plant growth and, consequently, to flower production. The strong association found between the yield and the number of flowers per plant allowed it to be deduced that the most important yield determinant in pyrethrum is the differentiation of flowers, and, after differentiation, further factors addressed to flower enlargement and increased size have lower relevance in yield assessment.

No significant differences were found between plants obtained by means of conventional cuttings and plants obtained through micropropagation.

Hence, this method allowed the simple and effective mass production of plantlets, a crucial step to encourage pyrethrum cultivation on a medium–large scale.

5. Conclusions

In this work, we compared the field performance of *Chrysanthemum cinerariifolium* plants produced by conventional cuttings and by a micropropagation procedure in order to evaluate if in vitro-produced plants could be a valuable material for a sustainable medium–large-scale production.

C. cinerariifolium has been cultivated in large areas of nearly all continents for the industrial extraction of pyrethrins. The production of secondary metabolites from plants could be problematic due to several factors such as the environment, variations in crop quality and losses in storage and handling. In this context, the production of secondary metabolites from cell cultures is an attractive proposal, and the availability of a reliable protocol for in vitro *C. cinerariifolium* propagation is an attractive prospective. The data presented underline that conventional and micropropagated plants of *C. cinerariifolium* are characterized by similar field performance, in both cases allowing long-lasting stands

with satisfactory yields, although reduced technical inputs were applied. In conclusion, micropropagated plants could be a valuable material for the medium–large scale production of plants to obtain flowers for pyrethrin extraction.

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