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Agrobacterium rhizogenes-Mediated Transformation in Mediterranean *Helichrysum*

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ABSTRACT

Helichrysum italicum (Roth) G. Don and *H. stoechas* (L.) Moench are aromatic wild species of the Mediterranean region. The plants grow in arid soils, from the cliffs above the sea to the hills, where they flower from May to August. The bright yellow flower heads contain essential oils, flavonoids (helichrysin A and B), tannins and caffeic acid. *Helichrysum* has been used in folk medicine because of its antibacterial, antitoxic, diuretic and antiallergic properties. The flowering stems are also used dried as "everlasting flowers". *A. rhizogenes* ATCC 15834 wild type strain was effective to induce hairy roots in leaf and root tissue of micropropagated *H. italicum* and *H. stoechas* Italian accessions. Hairy root-lines, originated from independent transformation events, were recovered. *H. stoechas* hairy root-regenerated plant lines, and their self pollinated progeny, have been studied for three years to assess their ornamental value and to test the antibacterial activity of the inflorescences extracts. The transgenic hairy root-derived plant lines showed distinct morphological and physiological growth patterns. Moreover, peculiar characters, induced by *A. rhizogenes* genes, were transmitted to the progeny.

Keywords: *Asteraceae*, everlasting, field trial, hairy root, *Helichrysum italicum*, *Helichrysum stoechas*, progeny

INTRODUCTION

The name *Helichrysum* (family *Asteraceae*) is derived by the Greek words *élios*=sun and *chrysòs*=gold, for the bright yellow colour of the flowers; the genus consists of about 600 species that occur in Africa (with 245 species in Southern Africa), Madagascar, Australasia and Eurasia. The Mediterranean *Helichrysum* species are perennial shrubs growing in arid soils, from the cliffs above the sea to the hills up to 600-800 m, where they flower from May to August. Cultivation is possible in a light, well-drained soil, in a sunny, sheltered position; the plants are intolerant of excessive moisture and are drought resistant. Propagation is by seeds and by cuttings of half-ripe wood (Cervelli 2005). Some Italian species are endemic to the island of Sardinia (*H. montelinasanum* Ed. Schmid) and Sicily (*H. barrelieri* (Ten.) Greuter, *H. hyblaeum* Brullo, *H. nebrodense* Heldr., *H. pendulum* (C. Presl) C. Presl, *H. rupestre* (Rafin) DC. var. errerae (Tineo) Pignatti) and are considered endangered species (Scoppola and Magrini 2005). The *Helichrysum* inflorescences have been used in folk medicine since Roman times, because of their antibacterial, antitoxic, diuretic and antiallergic properties; moreover the flowering stems are dried and used as "everlasting flowers", since they keep their shape and colour wholesome, for a long time.

H. stoechas (L.) Moench is a perennial shrub with rounded stems 5-90 cm high, wooded at the base, erect, bearing narrow soft leaves covered with white woolly hair. The scented flowers, 5-7 mm in diameter, are hermaphrodite and are collected in a compact corymb of yellow, globous capitula with external bracts double the length of internal ones.

H. italicum (Roth) G. Don, syn. *H. angustifolium* (Lam.) DC., cohabits in the same areas and flowers at the same time, but the whole plant smells of curry, especially after rain. The evergreen shrubs have erect corned stems 0.6-1 m high and linear narrow leaves 2-4 mm diameter. The flowers are collected in dense corymbs of conical capitula with external glabrous bud-scales, yellow straw-coloured and are pollinated by insects. The leaves are used as a flavouring in salads and cooked foods. The drug contains essential oils (0.05%), flavonoids (helichrysin A and B) and tannins and is used in cosmetic preparations devoted to moisturizing cream. The essential oil is used as a flavouring to enhance fruit flavours in sweets, ice cream, baked goods, soft drinks and chewing gum. A tea is made from the flower heads.

The volatile constituents of four *Helichrysum* species growing in Greece were analysed by Roussis *et al*. (2000) and the components of essential oils of *H. italicum* subsp. *italicum* growing in Corsica were investigated by Bianchini *et al*. (2001). *H. italicum* G. Don ethanolic extract antibacterial activity was tested against oral streptococci (Nostro *et al*. 2004). The *in vitro* antioxidant methanol extract activity was examined in four *Helichrysum* species (*Helichrysum noeanum* Boiss., *H. chionophilum* Boiss. & Bal., *H. plicatum* DC. subsp. *plicatum* and *H. arenarium* (L.) Moench. subsp. aucheri (Boiss.) Davis & Kuphicha) from Turkey (Bektas *et al*. 2005).

Tissue culture from *in vivo*-germinated seedlings was established in two Italian accessions *H. italicum* (Roth) G. Don and *H. stoechas* (L.) Moench. Shoot induction was obtained from micropropagated leaf tissue on a medium supplemented with zeatin and IAA (*H. italicum*) and on a medium with thidiazuron (*H. stoechas*), a system for callus production was developed by Giovannini *et al*. (2003). *A. rhizogenes* 15834 wild type strain was effective in inducing hairy roots in *H. italicum* and *H. stoechas* leaf and root tissues of micropropagated plants. The T-DNA *rolC* gene was detected by PCR analysis in *H. italicum* and *H. stoechas* hairy root lines. *H. italicum* hairy root liquid culture has been exploited for the production of *Helichrysum* secondary metabolites and for scaling-up in a bioreactor (Giovannini 2006).

Regeneration of viable plants through transformed hairy roots has been reported in some crop species (Christey 2001), in several ornamentals (Casanova *et al*. 2005) and in medicinal plants (Figueiredo *et al*. 2006; Guillon *et al*.

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2006). There are only few reports on hairy root-regenerated plants in woody species and shrubs (Kang *et al*. 2006).

H. stoechas (L.) Moench hairy root-regenerated plant lines exhibited typical alterations due to *A. rhizogenes* T-DNA gene expression: early flowering, reduced height, increased number of capitula per corymb, and a reduced flower stalk length (Giovannini *et al*. 2007). The transgenic *H. stoechas* plants and their self pollinated progeny have been studied to assess their improved ornamental and pharmaceutical value.

MATERIALS AND METHODS

A. rhizogenes-mediated transformation

Leaf and root tissues of *H. italicum* (Roth) G. Don and *H. stoechas* (L.) Moench micropropagated plant clones (Cl10 and Cl7 respectively), originating from the Botanical Garden of Siena (in Tuscany, Italy), were co-cultivated with a solution of *A. rhizogenes* wild type strain 15834 (American Type Culture Collection ATCC). The overnight-grown bacterial suspension was diluted 1: 10 (v/v) in sterile water ($OD_{550\ nm}$ = 0.1). Root pieces (10-20 mm long) and leaf fragments (30-40 mm^2) were cut from one year micropropagated plants (about 6 vegetative generations) and gently wounded with a lancet blade. Explants were soaked for 20 min in the bacterial suspension; control explants were soaked with sterile distilled water for the same time. Infected and control fragments were then placed on Petri dishes on a medium constituted by MS salts and vitamins (Murashige and Skoog 1962), 30 gl^{-1} sucrose, pH 5.7, added with 8 gl^{-1} Bacto agar (BM medium). After three days all the explants were transferred to BM medium supplemented with 100 mg l^{-1} Cefotaxime (BM-CX), according to Di Guardo (2003). Roots developed from cocultivated explants were excised and maintained on BM-CX medium by subculturing at 5-week intervals. Independently originated hairy root lines were selected on the basis of their characteristic phenotype of rapid branching, fast growth and white hair development on hormone-free medium. Molecular analysis was performed on total genomic DNA that was extracted and purified from 100 mg of hairy roots and micropropagated plant fresh root tissue, following the mini-DNeasy Plant Kit (Quiagen). The DNA amount was determined spectrophotometrically. *A. rhizogenes* 15834 DNA was extracted according to Klimyuk (1993). Each PCR reaction was performed in a final volume of 25 µl, 200 µM dNTPs, 0.3 µM of each primer, 1x PCR buffer, 1 unit of Taq DNA polymerase, 2.5 mM $MgCl_2$, and 80 ng of DNA template. PCR conditions for *rol*C fragment amplification were: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 60s, 65°C for 60 s, 72°C for 2 min and a final elongation step of 10 min at 72°C in programmable Thermal PTC-100TM controller (MJ Research, Inc, USA). PCR conditions for *virC1* fragment amplification were: 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 60°C for 1 min, 72°C for 30 s and a final elongation step of 7 min at 72°C. Amplification products were resolved by electrophoresis on 2% agar gel in TE buffer and visualised by ethidium bromide under UV light. Hairy root liquid cultures were established from hairy root tissue and were grown in 300 ml glass vases with 30 ml of BM-CX medium and cultured in the growth chamber, in both the dark and light, with continuous rotary shaking (78 rpm). Culture medium was substituted weekly with fresh medium.

Plant growth and *in vivo* experimental design

Transgenic plants regenerated from the *H. stoechas* hairy root lines B, E, M and N were micropropagated and subcultured monthly on Bacto agar-supplemented (8 g/l) BM-CX medium. The micropropagated plant clone Cl7 was used as the source of control (CT). About twenty rooted plants of each genotype were transferred to *in vivo* conditions in sterilized sand: soil (1:1 v/v) mixture consisting of neutral peat (20% total carbon – 0.5% nitrogen – 40% total organic matter) Tercomposti (Italy), autoclaved for 20 min at 121°C and common silica sand (Asplanato Vittorio, Italy), autoclaved for 20 min at 121°C, in filled perforated potting bags with 90 plants × unit area (1 m^2) and hardened, under mist (10 sec vaporization every 30 min) for 15 days. After hardening, plants were maintained in greenhouse at 23 ± 2°C/15 ± 2°C, with natural day length. The percentage of acclimatisation was evaluated after 15 days. The acclimatised plants were further propagated *in vivo* by softwood cuttings without using rooting powder and cultivated in 0.483 l pots, in the above sand: soil (1:1 v/v) mixture, in a naturally lit greenhouse at 23 ± 2°C/15 ± 2°C.

Potted plants derived from the hairy root lines E, M and N and from the control were used in the 2005 field trial. Line B plants were not sufficient for the trial. The experimental design was arranged in 4 randomized blocks with 15 pots per block and one plant per pot. From the end of April 2005, these data were collected for each pot: plant height and diameter, number and length of primary branches (ten branches for each pot). Flowering time was monitored by counting weekly the number of inflorescences (corymbs) at anthesis per plant. The corymb primary branch length and the number of capitula per corymb were measured from 30 inflorescences of each genotype.

Plants derived from the hairy root lines E, B, M and N and from the control were used in the 2006 field trial. The experimental design was arranged in 4 randomized blocks with 16 pots per block and one plant per pot. From the end of April 2006, these data were collected for each pot: plant height and diameter, number and length of primary branches (ten branches for each pot).

Inheritability of the characters in the progeny

Seeds collected in the 2005 experimental trial (open pollination) were sown in sandy soil. The seedlings were grown in 0.293 l pots, in sand: soil (1:1 v/v) mixture consisting of neutral peat (20% total carbon – 0.5% nitrogen - 40% total organic matter) Tercomposti (Italy) and common silica sand (Asplanato Vittorio, Italy), in a naturally lit greenhouse, at 23 ± 2°C/15 ± 2°C.

Plant height, primary branch number and length were collected three months from germination. The plants were further *in vivo* propagated by softwood cuttings, without using rooting powder, in 0.483 l pots. DNA analysis was performed in line E progenies to assess *A. rhizogenes rolC* gene segregation. Total genomic DNA was extracted and purified from 100 mg of *in vivo* fresh leaf tissue using the mini-DNeasy Plant Kit (Quiagen). The amount of DNA was determined spectrophotometrically and each PCR reaction was performed using the same conditions as described above.

Plants derived from line E, from line E self-pollinated progeny genotype E/1, E/3, E/4, E/5 and E/7 and from the control were used in the 2007 field trial. The experimental design was arranged in 3 randomized blocks with 14 pots per block and one plant per pot. From the end of May 2007, these data were collected for each pot: plant height and diameter, total number of inflorescences.

Antimicrobial activity of inflorescences

H. stoechas flowering stems of the hairy root-regenerated plant lines E, M and N and from the control were harvested after 240 days from potting (end of July 2006). The flowering stems were air dried at room temperature until constant weight. Established amount of the air dried inflorescences (10 g) was extracted with 100 ml of 95% ethanol (Merck, Sharp & Dome, S.p.A., Rome, Italy) at room temperature for 48 h. The extract was strained through filter paper, concentrated to dryness using a rotary evaporator at 37°C. A stock solution of the residue was made at a concentration of 200 mg ml^{-1} of 95% ethanol.

The microorganisms used in this study were: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* O901, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus mutans* ATCC 35668, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404 and *Fusarium oxysporum* wild type.

For the antimicrobial tests, Muller Hinton Agar (bacteria) and Sabouraud Dextrose Agar (mycetes) were used. For inocula, cells were grown overnight on Muller Hinton Broth (bacteria) and on Sabouraud Liquid Medium (yeasts). The moulds were cultures on Sabouraud Dextrose Agar in order to harvest the mature spores. All media were purchased from Oxoid.

Overnight broth cultures, adjusted to yield approximately 5.0

x 10^7 cfu ml^{-1} for bacteria, and 0.5 Mc Farland for *C. albicans*, were streaked with a calibrate loop on plates containing appropriate solid medium. For *A. niger* and *F. oxysporum,* suspensions of mature spores were obtained by washing gently the surface of solid media with a 0.05% solution of Tween 80 (Sigma-Aldrich) and the resulting suspension was adjusted to 70% transmission at 530 nm. Filter paper discs (6 mm diameter) (Oxoid) were placed on the inoculated agar surfaces and filled with 20 µl of *H. stoechas* stock solution.

Plates were incubated at 37°C for 18-24 h and at 30°C for 2-3 days for bacteria and mycetes, respectively. All determinations were performed in duplicate and the antimicrobial activity was expressed as the mean of inhibition zone diameters (including disc diameter of 6 mm) ± standard deviations. The minimum inhibitory concentration (MIC) of each strain was measured by the agar dilution method according to the National Committee for Clinical Laboratory Standard NCCLS guidelines (2000), using a multipoint replicator and delivering 1 µl of standardized microbial suspension. The final extract concentration ranged from 4000 to 3.9 µg ml^{-1}. Plates were incubated at 37°C for 18-24 h and at 30°C for 2-3 days for bacteria and mycetes, respectively. All determinations were performed in duplicate and the MIC was defined as the lowest concentration of extract inhibiting the visible growth.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Means were compared by a Student-Newman-Keul's multiple range test with $P \leq 0.05$.

RESULTS AND DISCUSSION

Hairy root induction

Helichrysum aromatic wild species and endemic taxa are peculiar to the Mediterranean region. The world consumption of herbal medicines is widespread and increased harvesting from the wild is causing loss of genetic diversity and habitat destruction. The use of tissue culture is a viable alternative and offers the opportunity to overcome the problems that are inherent in herbal extracts: misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. The use of controlled environments can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins (Canter *et al*. 2005). Micropropagation has been a useful tool for germplasm conservation and for multiplication of two *H. italicum* and *H. stoechas* Italian accessions (Giovannini *et al*. 2003); moreover *A. rhizogenes* wild-type strain was effective in inducing hairy roots in two *H. italicum* and *H. stoechas* micropropagated clones. Independently originated hairy root lines were selected on the base of their characteristic phenotype of rapid branching, fast growth and white hair development on hormone-free medium. *A. rhizogenes* T-DNA *rol*C gene was detected by PCR analysis in three *H. italicum* hairy root lines, originated from co-cultivated leaf tissues and four *H. stoechas* hairy root lines, originated from co-cultivated roots. Hairy root liquid cultures were established in *H. italicum.* Fast hairy root growth was observed in both the dark and light, in the absence of growth regulators (Giovannini 2006). Hairy root cultures can grow as fast as unorganized plant cell suspensions while maintaining a stable differentiated phenotype; furthermore secondary metabolite synthesis is not strictly limited to those that are normally produced in roots of differentiated plants (Kim *et al.* 2002). Antibacterial activity against the Gram-positive bacteria *Bacillus cereus*, *B. pumilus*, *B. subtilis* and *Staphylococcus aureus* as well as the Gram-negative bacterium *Serratia marcescens*, of a callus culture of an endemic South African species (*H. pedunculatum*) was reported by Dilika and Meyer (1998). *H. italicum* hairy root liquid cultures do provide a fast-growing active tissue with a biosynthetic potential that can be exploited for biological assays against pathogens and for bioreactor cultures, thus favouring the pharmaceutical, food additive and cosmetic industries for production of specific medicinal products (Figueiredo *et al*. 2006).

H. stoechas hairy root-regenerated plants

Shoots spontaneously developed from *H. stoechas* hairy root lines (B, E, N and M) on hormone-free medium in the light. Rooted plants were acclimatised in the greenhouse with a 66, 60, 45, 80 and 77.7% survival for the control (CT) and B, E, M, and N lines, respectively. The plants were further propagated *in vivo* by cuttings with 90% of rooting. The hairy root-regenerated plants were cultivated in

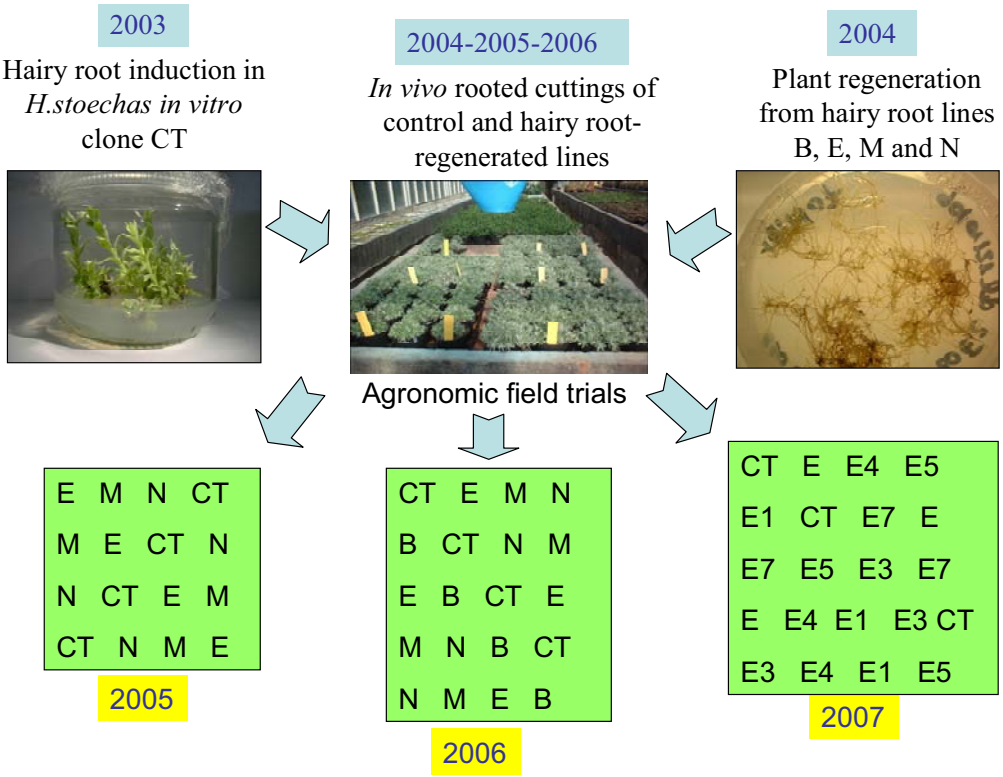

Fig. 1 *H. stoechas* hairy root-regenerated plants induction, agronomic trials and experimental design.

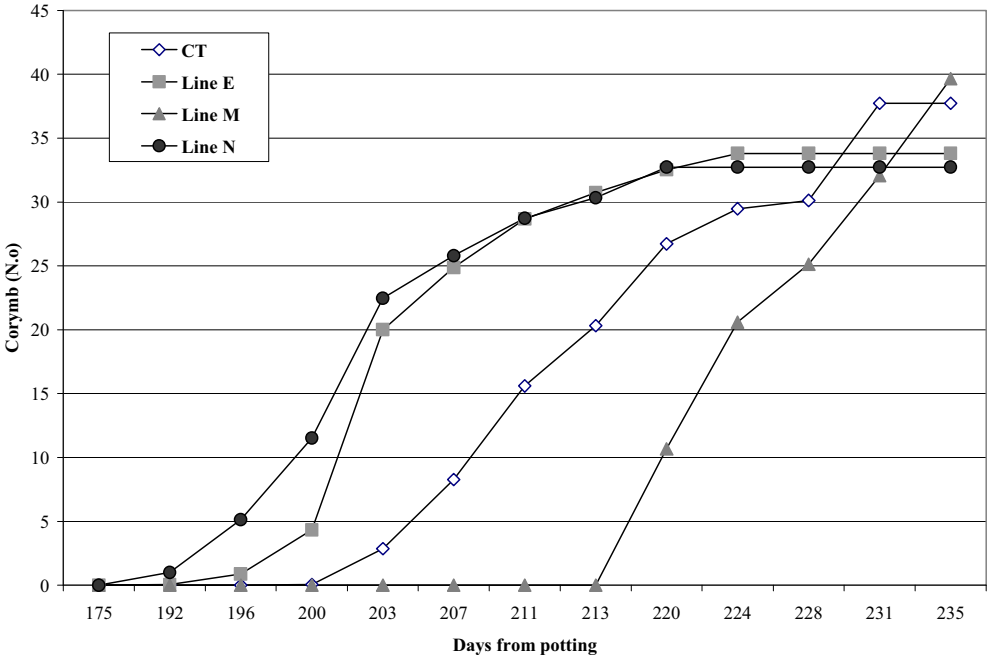

Fig. 2 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control plant flowering time: the number of inflorescence (corymb) at anthesis per plant was determined weekly.

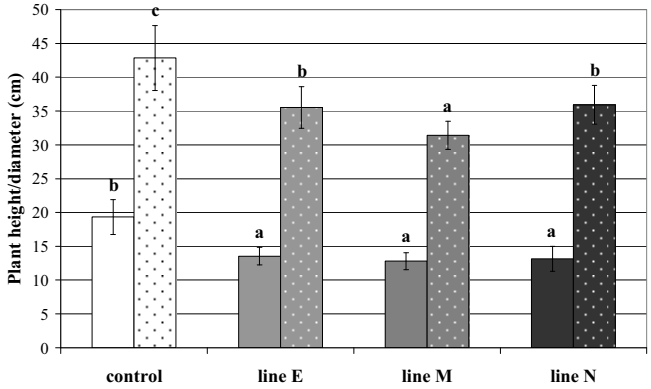

Fig. 3 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control plant shape: plant height (left) and plant diameter (right). Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

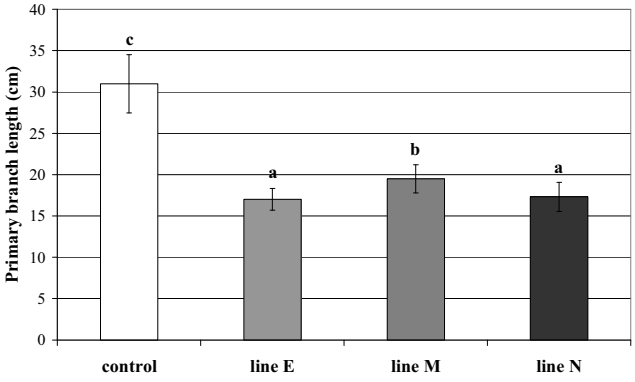

Fig. 5 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control plant primary branch length. Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

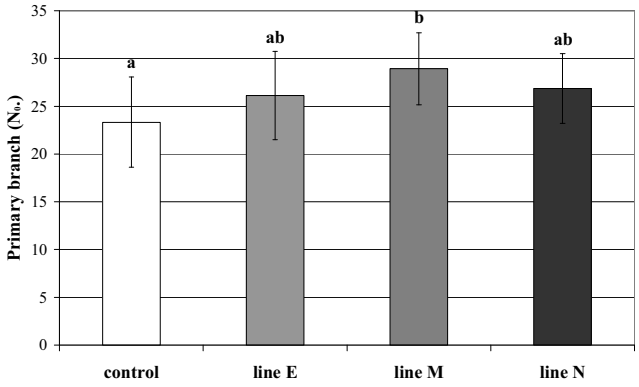

Fig. 4 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control plant primary branch number. Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

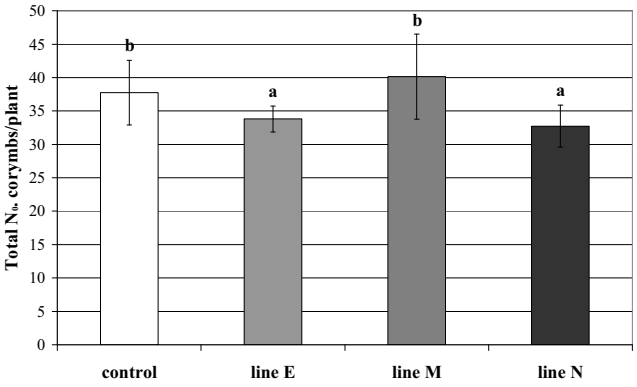

Fig. 6 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control total number of inflorescences (corymbs) per plant. Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

pots in agronomic field trials for three years to study morphological and physiological alterations (**Fig. 1**). In the 2005 agronomic field trial line E and N plants showed early flowering as compared to the control: they started to flower at 192 days from potting, 8 days before the control plants; on the other hand, line M plants started to flower 20 days after the control (**Fig. 2**). *H. stoechas* hairy root-regenerated plants lines E, M and N showed a more compact plant habit: plant height and plant diameter were significantly reduced compared to the control (**Fig. 3**). The number of primary branches was not affected in line E and N (**Fig. 4**), whereas primary branch length was significantly reduced

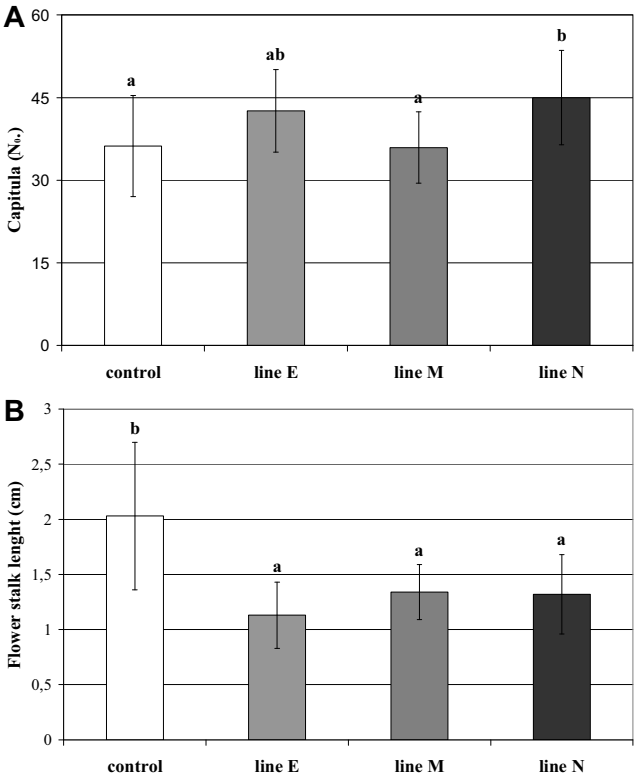

Fig. 7 Agronomic field trial 2005. *H. stoechas* hairy root-regenerated line E, M, N and control plant capitulum number per inflorescence (corymb) (**A**) and flower stalk length (**B**). Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

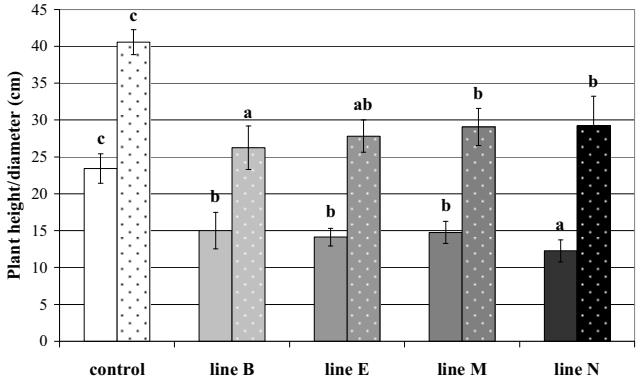

Fig. 9 Agronomic field trial 2006. *H. stoechas* hairy root-regenerated line B, E, M, N and control plant shape: plant height (left) and plant diameter (right). Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

(42%) in all hairy root-regenerated plant lines as compared to the control (**Fig. 5**). The total number of inflorescences (corymbs) per plant was significantly reduced in line E and N, as compared to the control and line M (**Fig. 6**). The capitulum number per corymb was not affected in line E and M, but was significantly increased (24%) in line N, as compared to the control (**Fig. 7A**). The flower stalk was significantly reduced in all the three lines (**Fig. 7B)**. Among the three independently originated *H. stoechas* transgenic genotypes, line N showed improved ornamental characters: early flowering, reduced height, increased number of capitula per corymb, and a reduced flower stalk length (**Fig. 8**). In the 2006 agronomic field trial the total number of inflorescences per plant was not significantly different among all the lines (control: 55.13 a; line B: 48.07 a; line E: 48.07 a; line M: 51.38 a; line N: 54.19 a). *H. stoechas* hairy root-regenerated plants showed a more compact plant habit: plant height and plant diameter were significantly reduced in B, E, M and N lines compared to the control (**Fig. 9**). The number of primary branches was significantly higher in line M (**Fig. 10**), whereas primary branch length was significantly reduced in all lines (**Fig. 11**). In the case of floricultural crops, molecular regulation of flowering and floral traits, as well as alterations in plant shape, via the application of *rol* genes, contributed to the improvement of the ornamental plants (Smith *et al*. 2006). Currently, the number of *rol* transgenic plants, in particularly *rol*C, continues to increase, and in many cases, beneficial traits have been obtained (Casanova *et al*. 2005; Smith *et al*. 2006). The *H. stoechas* hairy root-regenerated plants display some of the characters of the "hairy-root phenotype" imputed to *rolC*

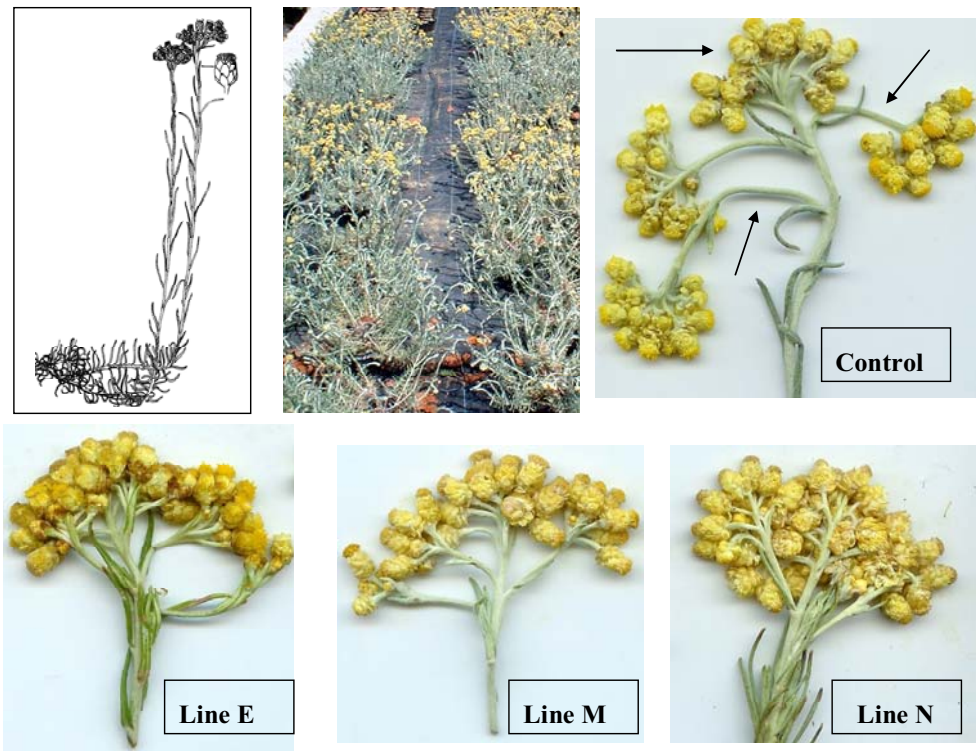

Fig. 8 Agronomic field trial 2005. *H stoechas* hairy root-regenerated line E, M N and control plant inflorescences (corymb): arrows indicate the corymb capitula and flower stalk in the control.

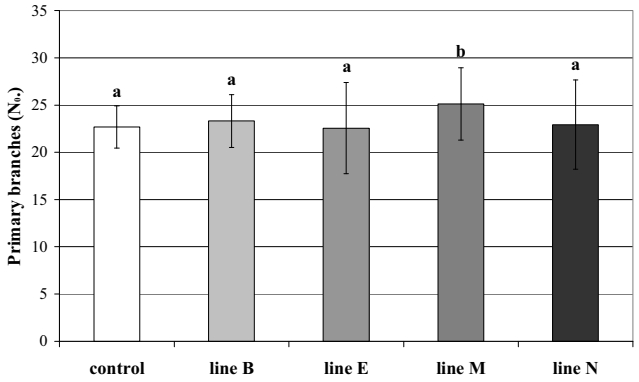

Fig. 10 Agronomic field trial 2006. Agronomic field trial 2006. *H. stoechas* hairy root-regenerated line B, E, M, N and control plant primary branch number. Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

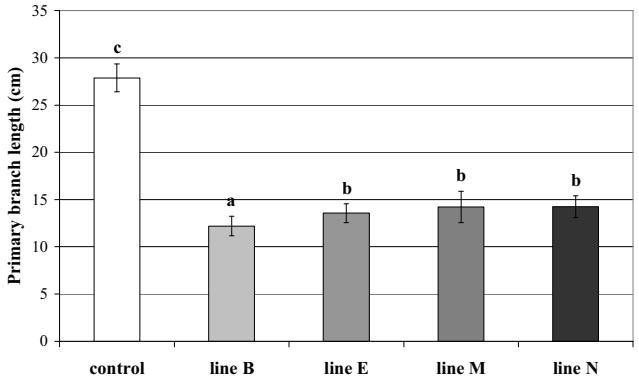

Fig. 11 Agronomic field trial 2006. Agronomic field trial 2006. *H. stoechas* hairy root-regenerated line B, E, M, N and control plant primary branch length. Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

Fig. 12 Agronomic field trial 2006. *H. stoechas* hairy root-regenerated plant line E, N, B, M and control plants (on the right) at flowering.

gene: dwarfness, the reduction of primary branch length that leads to a bushy phenotype, smaller inflorescences and advanced flowering in two lines (**Fig. 12**). However, further research is needed to determine the effects of *A. rhizogenes* T-DNA proteins and their interaction with other proteins, organs and environmental factors, in order to understand all the different phenotypical and biochemical effects they may produce in transgenic plants.

Inheritability of the characters in *H. stoechas* hairy root-regenerated line E progeny

The *A. rhizogenes rol*C gene was transmitted to line E progeny (four out of 8) and a reduced length of the primary branches was detected in the young *rol*C positive plantlets (**Table 1**). In the 2007 agronomic field trial the self pollinated progeny genotypes of hairy root-regenerated line E, which were positive for *A. rhizogenes rol*C amplification (E/1 and E/5), displayed some ornamental characters of the related transgenic line E (a reduced plant height), at the same time they showed a reduced number of inflorescences per plant. The genotypes negative for *A. rhizogenes rol*C amplification (E/3, E/4 and E/7) behaved more or less as the control plants (**Table 2**). In the natural genetic transformation by *A. rhizogenes* the wild-type Ri T-DNA, which causes root formation and which is compatible with plant regeneration, should be able to enter the germline in plants that regenerate naturally from their roots. Root-inducing T-DNA is not disadvantageous in some species as datura (Giovannini *et al*. 1997), tobacco (Hamamoto *et al*. 1991) and scented geranium (Pellegrineschi *et al*. 1994), but in other species, such as endive (Sun *et al*. 1991) or black locust (Han *et al*. 1993) it can interfere with essential plant functions. To compensate, the expression of this information becomes attenuated, e.g. through increasing ploidy level and methylation (Martin-Tanguy *et al*. 1996), or through segregation of truncated T-DNA insertions (Limami *et al*.

Table 1 *A. rhizogenes rolC* gene amplification and ornamental characters in the self pollinated progeny (one plant each genotype) of *H. stoechas* hairy root-regenerated plant line E, three months after seedling germination.

Line E progeny genotypes	*A. rhizogenes rol C* gene amplification	Plant height (cm)	Primary branches	
			Total №	Branch length* (cm)
E / 1	+	9	17	5.75 ± 2.64 (N=6)
E / 2	+	7.5	19	2.95 ± 2.40 (N=10)
E / 3	-	27.5	14	8.10 ± 3.64 (N=10)
E / 4	-	16	7	9.21 ± 2.67 (N=7)
E / 5	+	12.5	18	3.95 ± 2.73 (N=10)
E / 6	+	7.5	9	3.71 ± 2.03 (N=7)
E / 7	-	17.5	17	9.80 ± 7.47 (N=10)
E / 8	+	15.5	14	7.75 ± 3.60 (N=10)

*Mean number ± Standard Error

Table 2 Agronomic field trial 2007. Ornamental characters in the self pollinated progeny of *H. stoechas* hairy root-regenerated line E, line E, and control (CT) plants.

Genotype	Inflorescences (total №)	Plant shape	
		Height (cm)	Diameter (cm)
CT	44.90 ± 15.11 bc	24.25 ± 2.83 d	31.00 ± 3.59 cd
Line E	50.58 ± 7.08 c	17.25 ± 1.86 b	24.00 ± 3.30 a
E / 1	13.83 ± 6.16 a	15.00 ± 1.70 a	21.75 ± 2.13 a
E / 3	38.41 ± 6.57 b	23.75 ± 2.37 d	32.25 ± 4.22 d
E / 4	35.61 ± 9.56 b	20.75 ± 2.49 c	29.00 ± 3.38 bcd
E / 5	19.00 ± 11.19 a	17.75 ± 1.60 b	27.67 ± 3.28 bc
E / 7	43.41 ± 10.80 b	20.84 ± 2.20 c	27.17 ± 3.76 b

Means ± SE were compared by Student-Newman-Keuls multiple range test. Different letter indicates significant differences among treatments at $P \leq 0.05$.

Table 3 Antimicrobial activity of *H. stoechas* inflorescence extracts. Disc diffusion test: means of inhibition zone diameters (including disc diameter of 6 mm) ± standard deviations.

Micro-organisms	**Samples** (inhibition zone diameters, mm)			
	Control	**Line E**	**Line M**	**Line N**
Gram negative bacteria				
E. coli ATCC 25922	7.3 ± 1.1	6.6 ± 0.5	7.0 ± 1.-	6.6 ± 0.5
Pseudomonas aeruginosa ATCC 9027	6.6 ± 1.1	8.3 ± 1.5	6 ± 0.5	7.0 ± 1.0
Salmonella typhi O901	6.6 ± 1.1	8.0 ± 1.7	6 ± 1.1	7.6 ± 1.5
Gram positive bacteria				
Bacillus subtilis ATCC 6633	27.6 ± 2.1	27.6 ± 2.1	27.6 ± 3.6	27.0 ± 2.6
Listeria monocytogenes ATCC 7644	26.0 ± 3.6	27.6 ± 3.0	24.3 ± 2.1	26.6 ± 2.8
Staphylococcus aureus ATCC 6538P	28.6 ± 3.7	27.3 ± 5.8	27.6 ± 3.8	28.0 ± 5.2
Staphylococcus epidermidis ATCC 12228	30.3 ± 4.1	32.0 ± 5.3	33.3 ± 1.5	31.6 ± 3.5
Streptococcus mutans ATCC 35668	33.6 ± 4.7	31.6 ± 1.5	30.6 ± 1.5	32.3 ± 2.1
Mycetes				
Candida albicans ATCC 10231	-	-	-	-
Aspergillus niger ATCC 16404	-	-	-	-
Fusarium oxysporum wild type	-	-	-	-

Table 4 *H. stoechas* inflorescence extract biological activity. Minimum inhibitory concentrations (MICs).

Organisms	**Samples MIC (µg/ml)**			
	Control	**Line E**	**Line M**	**Line N**
Gram negative bacteria				
Escherichia coli ATCC 25922	>4000	>4000	>4000	>4000
Pseudomonas aeruginosa ATCC 9027	>4000	4000	>4000	>4000
Salmonella typhi O901	>4000	4000	>4000	4000
Gram positive bacteria				
Bacillus subtilis ATCC 6633	15.62	31.25	15.62	31.25
Listeria monocytogenes ATCC 7644	15.62	15.62-31.25	15.62	15.62-31.25
Staphylococcus aureus ATCC 6538P	31.25-62.50	31.25-62.50	31.25	62.50
Staphylococcus epidermidis ATCC	31.25	31.25	15.62	31.25-62.50
Streptococcus mutans ATCC 35668	7.81-15.62	7.81	7.81	7.81
Mycetes				
Candida albicans ATCC 10231	>4000	>4000	>4000	>4000
Aspergillus niger ATCC 16404	>4000	>4000	>4000	>4000
Fusarium oxysporum wild type	>4000	>4000	>4000	>4000

1998). Further research is needed to study Ri T-DNA insertions in *H. stoechas* hairy root-regenerated plant progeny.

Antimicrobial activity of *H. stoechas* inflorescences

The antimicrobial activity of ethanolic extracts of dried inflorescences was evaluated against three Gram-negative and five Gram-positive bacteria and against three mycetes by the disc diffusion test (**Table 3**) and the agar dilution method (**Table 4**). The transgenic E, N, and M lines and the control extracts exhibited antibacterial activity against the Gram-positive bacteria (MIC values 7.81-62.50 µg/ml). All samples were slightly active against the Gram-negative bacteria but not active at all against the mycetes *Candida albicans*, *Aspergillus niger* and *Fusarium oxysporum*. There were no significant differences among the samples. The hairy root-regenerated lines retained their antibacterial activity, mostly against *Staphylococcus aureus* and *Staphylococcus epidermidis*, suspected to be associated, in *H. fulgidum*, with a high percentage of essential oils (Bougatsos *et al*. 2004).

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REFERENCES

Bektas T, Munevver S, Askin A, Atalay S (2005) *In vitro* antioxidant activities of the methanol extracts of four *Helichrysum* species from Turkey. *Food Chemistry* **90**, 685-689

Bianchini A, Tomi P, Costa J, Bernardini AF (2001) Composition of *Helichrysum italicum* (Roth) G. Don fil. Subsp. *italicum* essential oils from Corsica (France). *Flavour and Fragrance Journal* **16**, 30-34

Bougatsos C, Ngassapa O, Runyoro DKB, Chinou IB (2004) Chemical composition and *in vitro* antimicrobial activity of the essential oils of two *Helichrysum* species from Tanzania. *Zeitschrift für Naturforschung C* **59c**, 368-372

Canter PH, Thomas H, Ernst E (2005) Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends in Biotechnology* **23**, 180-185

Casanova E, Trillasa MI, Moysseta LR, Vainstein A (2005) Influence of *rol* genes in floriculture. *Biotechnology Advances* **23**, 3-39

Cervelli C (2005) Le specie arbustive della macchia mediterranea, un patrimonio da valorizzare. Dipartimento Azienda Regionale Foreste Demaniali, *Collana Sicilia Foreste*, Palermo Italy, 26 pp

Christey, MC (2001) Use of Ri-mediated transformation for production of transgenic plants. *In Vitro Cellular and Developmental Biology – Plant* **37**, 687-700

Dilika F, Meyer JJM (1998) Antibacterial activity of *Helichrysum pedunculatum* callus culture. *South African Journal of Botany* **64**, 312-313

Di Guardo A, Cellarova E, Koperdakova A, Pistelli L, Ruffoni B, Allavena A, Giovannini A (2003) Hairy root induction and plant regeneration in *Hypericum perforatum* L. *Journal of Genetics and Breeding* **57**, 269-278

Figueiredo AC, Barroso JG, Pedro LG, Scheffer JJC (2006) Potentialities of hairy root cultures for *in vitro* essential oil production. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (Vol II), Global Science Books, Isleworth, UK, pp 478-486

Giovannini A, Pecchioni N, Rabaglio M, Allavena A (1997) Characterization of ornamental *Datura* plants transformed by *Agrobacterium rhizogenes*. *In Vitro Cellular and Developmental Biology – Plant* **33**, 101-106

Giovannini A, Amoretti M, Savona M, Di Guardo A, Ruffoni B (2003) Tissue culture in *Helichrysum* spp. *Acta Horticulture* **616**, 115-119

Giovannini A (2006) Tissue culture, cell culture and genetic transformation by wild type *Agrobacterium rhizogenes* in Mediterranean *Helichrysum*. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (Vol II), Global Science Books, Isleworth, UK, pp 222-226

Giovannini A, Mascarello C, Pipino L, Ruffoni B, Nostro A (2007) Caratterizzazione di piante di *H. stoechas* (L.) Moench rigenerate da hairy roots: architettura della pianta ed attività biologica. In: Paradossi A, Rognoni F, Mensuali A (Eds) *Colture Artificiali di Piante Medicinali, Produzione di Metaboliti Secondari Nelle Piante Medicinali in Coltura Artificiale*, Aracne S.r.l., Rome, Italy, pp 209-223

Guillon S, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Hairy root research: recent scenario and exciting prospects. *Current Opinion in Plant Biology* **9**, 341-346

Hamamoto H, Boulter ME, Shirsat AH, Croy EJ, Ellis JR (1990) Recovery of morphogenetically normal transgenic tobacco from hairy roots co-transformed with *Agrobacterium rhizogenes* and a binary vector plasmid. *Plant Cell Reports* **9**, 88-92

Han KH, Keathley DE, Davis JM, Gordon MP (1993) Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L. black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. *Plant Science* **88**, 149-157

Kang HJ, Anbazhagon VR, You XL, Moon HK, Yi JS, Choi YE (2006) Production of transgenic *Aralia alata* regenerated from *Agrobacterium rhizogenes*-mediated transformed roots. *Plant Cell, Tissue and Organ Culture* **85**, 187-196

Kim Y, Wyslouzil BE, Weathers PJ (2002) Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cellular and Developmental Biology – Plant* **38**, 1-10

Klimyuk VI, Carroll BJ, Thomas CM, Jones JDG (1993) Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal* **3**, 493-494

Limami MA, Sun L-Y, Douat C, Helgeson J, Tepfer D (1998) Natural genetic transformation by *Agrobacterium rhizogenes*. *Plant Physiology* **118**, 543-550

Martin-Tanguy J, Sun L-Y, Burtin D, Vernoy R, Rossin N, Tepfer D (1996) Attenuation of the phenotype caused by the rootinducing, left-hand, transferred DNA and its *rolA* gene. *Plant Physiology* **111**, 259-267

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

National Committee for Clinical Laboratory Standard (2000) In: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7 - A5. National Committee for Clinical Laboratory Standard, Wayne, PA

Nostro A, Cannatelli MA, Crisafi G, Musolino AD, Procopio F, Alonzo V (2004) Modification of hydrophobicity, *in vitro* adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Letters in Applied Microbiology* **38**, 423-427

Pellegrineschi A, Damon JP, Valtorta N, Paillard N, Tepfer D (1994) Improvement of ornamental characters and fragrance production in lemon-scented geranium through genetic transformation by *Agrobacterium rhizogenes*. *Biotechnology* **12**, 64-68

Roussis V, Tsoukatou M, Petrakis PV, Chinou I, Skoula M, Harborne JB (2000) Volatile constituents of four *Helichrysum* species growing in Greece. *Biochemical Systematics and Ecology* **28**, 163-175

Scoppola A, Magrini S (2005) The Italian vascular flora: references and sources. In: Scoppola A, Magrini S (Eds) *Atlante delle Specie a Rischio di Estinzione*, Palombi Editori, Rome, Italy, pp 47-67

Smith AG, John KE, Gardner N (2006) Dwarfing ornamental crops with the *rol*C gene. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (Vol II), Global Science Books, Isleworth, UK, pp 54-59

Sun L-Y, Touraud G, Charbonnier C, Tepfer D (1991) Modification of phenotype in Belgian endive (*Cichorium intybus*) through genetic transformation by *Agrobacterium rhizogenes*: conversion from biennial to annual flowering. *Transgenic Research* **1**, 14-22