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Micropropagation and *In vitro* Culture of Pyrethrum [*Chrysanthemum cinerariifolium* (Trev.) Vis.]

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ABSTRACT

Pyrethrum [Chrisanthemum cinerariifolium (Trev.) Vis. = Tanacetum cinearíaefolium (Trev.) Schultz-Bip.] is a perennial herbaceous plant belonging to the family Asteraceae, native to Albania and the area of former Yugoslavia. It is the only species in the genus Tanacetum having an agronomic importance, although the genus consists of several species producing similar types of bioactive metabolites. The species is grown in order to obtain the insecticidal compounds collectively termed pyrethrins, which are found primarily in the flower heads.

In this work we discuss the results found from a worldwide literature review about the micropropagation techniques followed on Pyrethrum, the in vitro culture conditions, and the ex vitro establishment trials under Mediterranean environmental conditions. Many technical problems concerning the propagation of the species seem to have been solved, and detailed protocols are available for an easy and fast propagation by seeds, vegetative splits, stem cuttings (rooted under mist or not), and tissue culture. The first attempts to introduce its cultivation into the semi-arid Mediterranean environments have brought to satisfactory results, and the species may be suggested as a valuable opportunity for the development strategies in new Mediterranean farming systems.

Keywords: Embriogenic callus, Mediterranean environments, Natural insecticides, Pyrethrins, Vegetative propagation.

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Pyrethrum as an Industrial Crop

The wide family of industrial crops includes all those plants that, directly or throughout the active compounds contained inside them, possess some medicinal or aromatic interest, or that may be addressed to many different uses (for cosmetic, dye, insecticidal purposes, or else). Among them, it is worth to include pyrethrum [*Chrysanthemum cinerariifolium* (Trev.) Vis.], a plant that was defined "Paramedicinal" (da Silva *et al.*, 2004), known since very ancient times due to its excellent insecticidal properties.

The species possesses interesting economical potentialities, in that it is addressed to chemical industry for the manufacturing of natural pyrethrins-based items. Despite the possibility to obtain synthetic products (Pyrethroids) that are strictly similar to natural pyrethrins, the interest towards this natural product is still very high, and the market potentialities that is possible to detect are significant.

Pyrethrum as a Crop in Mediterranean Environments

All the major world organizations (UN, FAO, EU) agree in recognizing to the multifunctionality of agriculture a crucial role for promoting a real and sustainable development of rural areas (Carrubba *et al.*, 2008). In many Mediterranean environments, that are often characterized by marginality conditions, the setting out of farming models based on such criteria is however especially difficult.

According to many experimental outcomes, a large number of pedo-climatical and socio-economical factors that confer to a territory its "marginality" conditions, may be effectively overpassed throughout the cultivation of some special industrial crop. In this scenario, the introduction of a new crop such as pyrethrum, seems to be an adequate choice in order to promote a sustainable and integrated development of rural areas, with a special reference to marginal ones. The cultivation of pyrethrum in some semi-arid environments of inner Sicily has led to interesting yields, both as total biomass and as flowers yield, even in lack of significant technical inputs (Carrubba and Ascolillo, 2009).

Origin, Importance and Diffusion of the Plant

Pyrethrum [*Chrysanthemum cinerariifolium* (Trev.) Vis.] is native to Albania and Dalmatia, a region in former Yugoslavia (Heywood, 1976).

The species is cultivated for the production of pyrethrins, that are a family of substances endowed with excellent insecticidal properties. Such properties were accidentally discovered in 1840 in Dalmatia and the large-scale cultivation and marketing of the species began in 1860 (McLaughlin, 1973).

In 1882 pyrethrum was introduced in Japan, that between the two World Wars became the major pyrethrum world producer (Purseglove, 1982). About in the 20s, also Switzerland and France became pyrethrum producers; in the same period, seeds of pyrethrum coming from Switzerland and Japan arrived to United Kingdom at the Rothamsted Experimental Station, and from there they moved to Kenya (Tuikong, 1984; Wandahwa *et al.*, 1996). Later on, also India, Tasmania, China, USA and many South America countries became pyrethrum producers. After the second World War,

the export from Japan stopped, and Kenya became the major pyrethrum world producer (Wandahwa *et al.*, 1996).

By now, the world area invested with pyrethrum is about 29.000 ha, with a total production of dry flowers almost reaching 9000 tons per year (FAO, 2013); the major pyrethrum sellers on the world market are the "Pyrethrum Board of Kenia" (PBK) and the "Botanical Resources Australia", located in Tasmania, Australia (Greenhill, 2007; MacDonald, 1995). In Europe, pyrethrum is grown in many countries, including Austria, France, Hungary, Spain and Russia; yields of 200-300 tons were registered in 2010 also in Italy.

Botanical Classification and Genetic Traits

Pyrethrum [*Chrysanthemum cinerariifolium* (Trev.) Vis. = *Tanacetum cinearíaefolium* (Trev.) Schultz-Bip.] is a perennial herb belonging to the family *Asteraceae*. According to the earlier botanical classification, the species belongs to the genus *Chrysanthemum*, whereas in a recent classification aiming to the simplification of this genus, pyrethrum was moved to the genus *Tanacetum* (Soreng and Cope, 1991).

The genus *Tanacetum* is one in more than 100 genera of the tribe *Anthemideae* (Soreng and Cope, 1991), that collects about 10 per cent of genera and 15 per cent of species belonging to the family *Asteraceae* (Heywood and Humphries, 1977). By far, the genus *Tanacetum* includes between 70 and 150 species, with the exact number varying according to the adopted classification (Abad *et al.*, 1995; Soreng and Cope, 1991; Heywood and Humphries, 1977). Hence, it results one of the largest genera inside the *Anthemideae* (Abad *et al.*, 1995).

The base chromosome number of pyrethrum, as found in other species of the tribe *Anthemideae*, is 2n=2x=18 (MacDonald, 1995; Heywood and Humpries, 1977; Virrankoski and Sorsa, 1968). Triploid (2n=3x=27) and tetraploid (2n=4x=36) clones have been however identified (MacDonald, 1995; Ottaro, 1977). Similarly to other *Compositae* (Leto *et al.*, 1994), the ploidy level influences the morphological traits: triploid pyrethrum plants show, as a matter of fact, larger flowers, longer stems and larger (although fewer) stomata with respect to diploid plants (Ottaro, 1977).

Many plant traits that are variously related to productivity are hereditary: number of flowers per plant (Singh and Sharma, 1989; Singh *et al.*, 1987; Singh *et al.*, 1988), average weight of flowers (Singh *et al.*, 1988), flowers size (Parlevliet, 1975; Parlevliet and Contant, 1970), production of pyrethrins (Singh and Singh, 1996).

Plant Description, Biology and Environmental Requirements

Within the Raunkiaer system, that categorizes plants using their diverse life-forms, pyrethrum is classed as a partially woody Chamaephyte.

The plant reaches 80-100 cm in height and is branchy, silvery green, often covered with a fine silky hair. The leaves are subdivided, with pinnate or palmate portions; in the basal leaves, 10-20 cm in length, blade is shorter than petiole, whereas the superior leaves are generally smaller and have a shorter petiole (Greenhill, 2007).

Optimum temperatures for photosynthesis range between 15 and 20°C. The vegetative period lasts several months, until flower differentiation, that occurs

irrespective of long or short photoperiodic conditions. The onset of flowering is induced by the occurrence of about six weeks at temperatures lower than 17°C (FAO, 1978; Glover, 1955; Roest, 1976). The alternance of night temperatures below 13 °C and day temperatures between 15 and 20 °C may enhance flowers yield (Roest, 1976).

The daisy-like pyrethrum inflorescences (capitula) have a 40-50 mm diameter. Inflorescences are lonely and brought on long flower stems; they bear two kinds of flowers, the tubular, hermaphrodite and yellow flowers in the central disk, and the ligulate, feminine and white flowers in the peripheral part of inflorescence (Brewer, 1968; Cantele, 2001; Greenhill, 2007) (Figure 9.1).

Pyrethrum is allogamous and pollinated by insects (Cantele, 2001). Both the disk and the ray flowers form the fruits (achenes), 2.5-3.5 mm long, located on the receptacle (McLaughlin, 1973) (Figure 9.2).

The species may be propagated both by seeds and by vegetative splits or stem cuttings. Pyrethrum seeds however germinate very slowly, and germination is uneven and reduced at high temperatures (30-35 °C) (Haque *et al.*, 2006). The treatment of seeds with ethrel and their moist pre-chilling improved the germination rate (46-80 per cent) (Haque *et al.*, 2006). Seed germination and seedling growth were inhibited by increasing salt and drought stress. Exposure to light could reduce germination from 52 per cent to 22 per cent and increased the mean germination time (MGT) from 7 to 12 days (Li *et al.*, 2011).

Hydropriming is an effective tool to improve the quality of pyrethrum seeds (Li *et al.*, 2011).

Pyrethrins are mainly stored inside small oil glands located on the external surface of achenes (93,7 per cent) (Bhat and Menary, 1986; Greenhill, 2007); lower quantities may be found in the disk flower (2,0 per cent), in the ray flowers (1,7 per cent) and in the receptacle (2,6 per cent) (Head, 1966).

Figure 9.1: Pyrethrum flowers at full blooming.

Table 9.1: Flower development stages of Pyrethrum and their duration from the beginning of stage 1 (Head, 1966; Wandahwa *et al.*, 1996).

Stage	*Description*	*Duration (Daya)*
1	Flower buds (closed)	0
2	Vertical ray flowers	12
3	Horizontal ray flowers, first row of disk flowers opened	16
4	About 3 rows of disk flowers opened	19
5	All disk flowers opened and ripe	21
6	Beginning of flowers decay	31
7	Full flowers decay	43
8	Dried stems 1 cm below flowers; harvest of seeds	60

Phyrethrins content is subjected to variations due to the development stage of capitula (Table 9.1). Their concentration, actually, shows an increment starting from the stage 1 (flower buds) until the maximum level, achieved when 3-4 rows (about ¾) of the disk flowers are opened (between stages 4 and 5), before the fall of ligulate

Figure 9.2: Pyrethrum seeds and dried flowers.

flowers (Bhat and Menary, 1984; Cantele, 2001; Wandahwa *et al.*, 1996); later on, the pyrethrins level gradually decreases (Wandahwa *et al.*, 1996). The concentration of isoprenoids (particularly pyrethrins) in pyrethrum depends, moreover, on plant genetic characters (Jones, 1968; Tedone *et al.*, 2004): many clones reach, for example, their highest pyrethrins content between stages 5 and 7 (Ikaku and Ngugi, 1989).

In Kenya, pyrethrum grows at high elevations, between 1500 and 3000 m a.s.l. (Wandahwa *et al.*, 1996). For its best development, the plant requires a high rainfall amount, between 1000 and 1400 mm per year (Cantele, 2001; Wandahwa *et al.*, 1996). A dry period, at least two months long, allows a "rejuvenation" of plant (Wandahwa *et al.*, 1996) and a better weeds control (Cantele, 2001). The crop, however, fails in environments with a prolonged dry period (7 months or more) (Wandahwa *et al.*, 1996).

Pyrethrum grows well on fertile, deep and well-drained soils (Wandahwa *et al.*, 1996), but it may find optimal growth conditions also on skeletal soils, slightly alkali or saline or calcareous (Kroll, 1963).

Pyrethrins and their Action Mechanism

Pyrethrins belong to the family of compounds known as "isoprenoids", formed by glucose and acetyl-CoA, whose structure is formed by a hydrocarbon skeleton with 5 carbon atoms (isoprene); in particular, they belong to the group of monoterpenes. Their most studied biosynthetic pathway is the "mevalonate pathway", that drives to the formation of the isoprene active unit with 5 carbon atoms (IPP) (Lichtenthaler *et al.*, 1997).

Generally speaking, pyrethrins are a group of six monoterpene esters, obtained throughout the esterification of two acids and three alcohols (pyrethrolone, jasmolone and cinerolone). The chrisantemic acid (monocarboxylique) is the basic isoprenoid for the esters named pyrethrin I, cinerine I and jasmoline I, collectively known as group I pyrethrins. At the same way, the pyrethric acid (bicarboxylic) is the basic isoprenoid for the pyrethrins of the group II (pyrethrin II, cinerine II and jasmoline II) (Godin *et al.*, 1963; Hitmi *et al.*, 2001).

According to Bhat (1995), the insecticidal activity of the pyrethrum extract is linked to the ratio between pyrethrins of the I and II group; due to their higher stability, pyrethrin I have a higher "kill-effect" *i.e.*, a proper insecticidal action, whereas pyrethrin II perform a quick "knock-down" effect on insects population (Bruneton, 1995; Cantele, 2001).

The raw extract of pyrethrum flowers contains about 30-35 per cent of pyrethrins (Table 9.2), along with small quantities of other isoprenoids, including carotenoids (0.82 per cent), chlorophylls (0.1 per cent) and taxasterols (5.0 per cent).

Pyrethrins are widely used all over the world as natural insecticides. Their importance is due to a number of typical traits that make them an ideal tool for pest control, above all in organic management (Wei *et al.*, 2006). They are actually useful against a high number of flying insects (Odinga and Angedu, 2003), including aphids, beetles, cycalins, thrips, whiteflies and Lepidoptera, determining reduced resistance phenomena.

Table 9.2: Composition of pyrethrins in pyrethrum dry flowers and extract. (from: Keskitalo, 1999; Head, 1966).

Compound	*Concentration per cent*	
	1	*2*
PYRETHRINS:	**2.00**	**30-35**
Pyrethrins I:	**0.92**	**14.8**
Cinerine I	0.18	2.2
Jasmoline I	0.09	1.2
Pyrethrine I	0.65	11.4
Pyrethrins II:	**1.08**	**15.2**
Cinerine II	0.26	3.5
Jasmoline II	0.10	1.2
Pyrethrine II	0.72	10.5

1 Concentration in dry flowers, per cent.

2 Concentration in raw extract from flowers, per cent.

Pyrethrins exert their activity above all by contact, throughout an action on the insect nervous system; even with sub-lethal doses, insects are paralyzed after few minutes or even in some seconds. The preferred ways of entrance are antennae, cerci, legs, and, above all, tracheal spiracles; once entered the insect, pyrethrins work on nervous cells (neurotoxic effect), provoking muscle contractions and uncoordinated movements of legs and wings, until complete paralysis (Gruppo SGD, 2010).

The rate at which of the active principle is metabolized is determined by the activity of the enzymes esterase and oxydase. The short duration of the toxic effect of pyrethrins, however, may also not to kill the insect. For this reason, the majority of naturally-based insecticides are supplemented with synergistic substances; these are non toxic substances, without any direct insecticidal effect, that are able to improve the adsorption of pyrethrins into the insect's body, inhibiting the action of the enzymes above. Among these substances we may list sesamine (a component of sesame oil) and PPB (piperonil-butoxyde), that, added in the same quantity as pyrethrum, enhance its toxicity upto 5 folds.

Pyrethrins are moreover characterized by a high repellant (insectifuge) and flushing-out action: the insects, disturbed by the insecticide, tend to leave their shelters, enhancing the possibility to meet the active compound. The strong repellant activities of pyrethrins are probably the main reason of their low toxicity at ingestion, since they may be easily regurgitated. Pyrethrins are not selective, since they act on all insects, irrespective they are noxious or useful. They are especially effective against insects that are not endowed with a resistant exoskeleton, such as potato beetle or bugs.

Due to its wide activity range, pyrethrum should be better used throughout treatments limited in time and localized on breeding ground of pests, minimizing the

effect on useful insects. It is preferable moreover to avoid interventions at blooming, to save the pollinating bees (Roviglioni, 2008).

Among the advantages of pyrethrins, with respect to all the other insecticides, the low toxicity for mammalians and other hot-blooded animals may be taken into account (Hitmi *et al.*, 1998), although they are highly toxic for fishes, reptiles and amphibians (and therefore their use must be avoided in proximity to rivers or lakes).

The products based on natural pyrethrins are thermo- and photolabile, hence they have the advantage not to leave noxious residual, decaying in a short time after the treatment (Gruppo SGD, 2010). With respect to plants, pyrethrins are not strictly systemic either cytotropic. After the treatment, it is enough to wait for 2 days before eating treated vegetables and fruit, and 3 days before casting useful insects in field (Roviglioni, 2008).

Pyrethrum *In vitro* Growing Techniques

Micropropagation

The traditional vegetative propagation methods often give unsatisfactory results for pyrethrum, that possesses a low multiplication rate (Hitmi *et al.*, 1998a). An additional problem is represented by the high susceptibility of plant to the root nematodes during the multiplication phases (Mwamba, 1996).

Hence, the necessity showed up to study the best conditions for the *in vitro* growing techniques of pyrethrum, with the aim to obtain, by means of the proper biotechnological strategies, an effective regeneration system.

In consideration of the industrial purposes of pyrethrum, the pointing out of these techniques may play, moreover, an important role in experiments of genetic manipulation (Hedayat *et al.*, 2009).

The achievement of optimal conditions for the *in vitro* growing of pyrethrum and other *Asteraceae* is rather difficult, so that these are often considered "recalcitrant" species. Such difficulties may be associated to phenomena of tissues browning, vitrification or bacterial contaminations (Keskitalo *et al.*, 1999), and they may be overpassed only with a proper *in vitro* "domestication" period (Keskitalo *et al.*, 1998; Keskitalo, 1999).

Bacterial contamination may be especially difficult to eliminate, above all when the explants to be introduced *in vitro* come from perennial plants or from plants that were however grown in open field. The problem is especially severe above all when it concerns the xylematic system, protected by the surface sterilization procedures (Hallmann *et al.*, 1997). The endophytic bacteria have probably developed complex interactions with host plants inside their co-evolutionary processes (Spencer, 1988), and such interactions are reasonably able to influence the physiology of host plants (Misaghi and Donndelinger, 1990), even without the outcoming of symptoms. Under special stress conditions, such as those shown up *in vitro*, however, also latent bacteria may turn pathogens, compromising crop growth and development (Leifert *et al.*, 1992).

Table 9.3: Protocols used for the disinfection of pyrethrum explants for *in vitro* establishment (Carrubba *et al.*, 2008a).

Steps	*I*	*II*	*III*	*IV*	*V*	*VI*
1°	Washing (water)	Washing (soap and water) (Tween 20)	Washing (soap and water) (Tween 20)	Washing (soap and water) (Tween 20)	Washing (soap and water) (Tween 20)	Washing (soap and water) (Tween 20)
2°	Ethanol 75 per cent (3 min)	HCl 1mol L^{-1} (few sec.)	Ethanol 75 per cent (3 min) + PVP at 1 per cent	Ethanol 75 per cent (40 sec)	Ethanol 75 per cent (40 sec)	Ethanol 75 per cent (40 sec)
3°	15 per cent NaClO (15 min)	15 per cent NaClO (20 min)	20 per cent NaClO (25 min) + PVP at 1 per cent	20 per cent NaClO (20 min) + PVP at 1 per cent	20 per cent NaClO (15 min) + PVP at 1 per cent	15 per cent NaClO (15 min) + PVP at 2 per cent
4°	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet	3 rinsings (5 min each) with sterile distilled water, under a Laminar Flow Cabinet

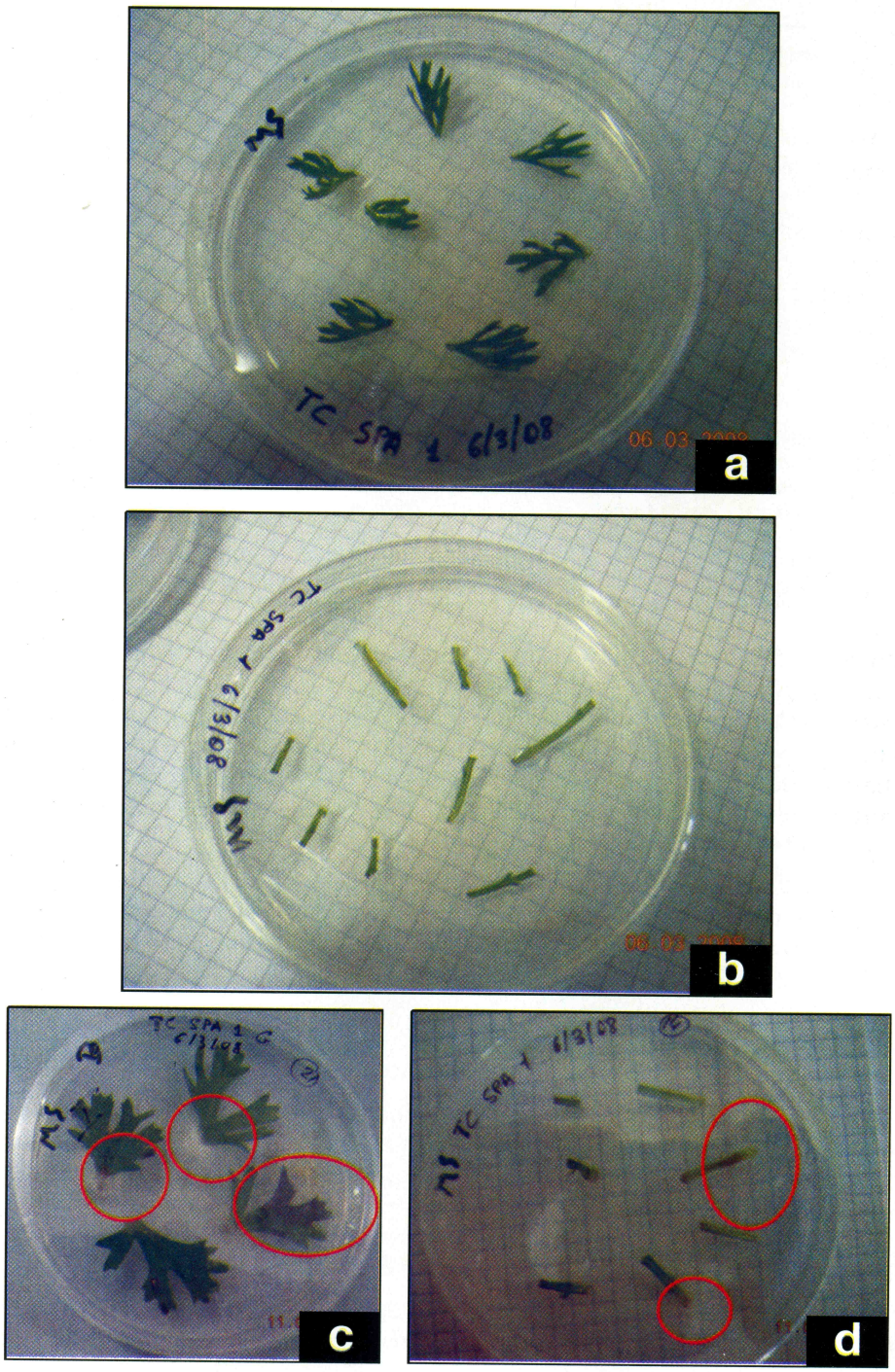
The term "vitrification" indicates, in tissue culture, a special morphological response of plant tissues under stress conditions (Franck *et al.*, 1995). The vitrified leaves show cell hypertrophy (Olmos and Hellin, 1998) and large intercellular spaces; tissues are less woody and vascular system is not normal (Gaspar *et al.*, 1987). From the biochemical point of view, in vitrified tissues the activity of many enzymes is altered. These symptoms may be reduced by modifying the components of the growth medium and the growth *in vitro* conditions.

Finally, the browning of *in vitro* tissues, is often associated to woody and perennial species, due to special enzymatic reactions, that lead to the oxidation of phenolic substances (Block and Lankes, 1995), or to external factors (presence of pathogens, high salt concentration, auxins or saccarose) (Curtis and Shetty, 1996; Jin *et al.*, 1996; Mohamed and Jayabalan, 1996; Choi *et al.*, 1998). The addition of some components in the growth medium may determine the reduction or elimination of tissue browning (Housti *et al.*, 1992) (Figures 9.3 a, b, c and d).

Satisfactory results (Carrubba *et al.*, 2008a) have been achieved by means of a continuous improvement of sterilization protocols of the explants (Table 9.3); the tissue browning, firstly detected on leaves and, above all, on shoots, was no longer observed starting from the application of the sterilization protocol III, by means of the addition of PVP (polyvinyl pyrrolidone; Housti *et al.*, 1992), whose concentration was brought up to 2 per cent in order to obtain the best results.

The procedure for a fast *in vitro* multiplication (micropropagation) of pyrethrum clones mostly uses as a basic medium the Murashige and Skoog (1962) (MS), with

Figure 9.3: Pyrethrum growing *in vitro*; leaves (a) and stem portions (b) at the beginning of growth; contaminations after 5 days of *in vitro* culture on leaves (c) and stem portions (d).



addition of various kinds and blends of phytoregulators. The MS medium allows, actually, to obtain the highest number of sprouts per explant, achieving high average values both for their length (mm) and for their weight (Hedayat *et al.*, 2009). Plants regeneration has been obtained starting from apical buds (Zito and Tio, 1990), axillary buds (Wambugu and Rangan, 1981), callus (Zito and Tio, 1990; Pal and Dhar, 1985), leaves and petiole segments (Hedayat *et al.*, 2009).

According to some authors (Liu and Gao, 2007), the highest *in vitro* multiplication rate may be obtained on the MS medium supplemented with 0.3 mg/l BA and 0.3 mg/l NAA. Other authors (Hedayat *et al.*, 2009) otherwise suggest higher concentrations (1.5 and 2 mg/l BA and NAA respectively) for the *in vitro* pyrethrum multiplication. In any case, the authors above claim that the combination of a cytochinine and an auxine is necessary in order to obtain a good cell multiplication. Similar results are reported by several other authors (Hitmi *et al.*, 1998; Pal and Dhar, 1985).

For *in vitro* rooting of pyrethrum sprouts, the best results have been obtained on the B5 medium (Gamborg *et al.*, 1968) with 2 mg/l NAA, that allows a 100 per cent rooting (with an average of 16 roots per sprout) in 3 weeks. Many other authors, however, have reported the possibility to obtain good *in vitro* rooting rates on a MS medium without any growth regulator (Catalano *et al.*, 2011; Hitmi *et al.*, 2001; Pal and Dhar, 1985).

A trial addressed to study pyrethrum roots formation without growth regulators was carried out on shoots that had been previously obtained *in vitro* (Catalano, 2010) (Figure 9.4). The multiplication was carried out in rooms at controlled temperature set at 22 ± 1 °C, under a 16-h photoperiod of 30 µmol $m^{-2}s^{-1}$ of irradiance. Roots formation began in about 3-4 days, reaching 100 per cent rooting in 8-10 days. According to Liu and Gao (2007), the growth of sprouts was measured at the beginning of the experiment and after 30 days, obtaining the Growth Rate of Sprouts (GRS) by means of the formula:

GRS = (30-days mass – initial mass)/initial mass ($g\ g^{-1}$);

as shown, the 30-days biomass accumulation was about 95 per cent with respect to the initial value (Table 9.4).

The growth of roots (Table 9.5) was evaluated by measuring their number, length, and mass in 30 days from the beginning of the experiment. In many cases a high number of roots (up to 11) was counted, and all the rooting system showed a uniform and vigorous development. The rooted plantlets were ready for the *ex vitro* transfer after 30-45 days.

Table 9.4: Growth rate of micropropagated pyrethrum shoots (Catalano, 2010). Mean values ± standard deviation (n=10).

Initial Fresh Mass (g)	*Fresh Mass after 30 days (g)**	*Growth Rate of Shoots (GRS)*
0.16 ± 0.09	0.31 ± 0.09	1.23 ± 0.73

* only the vegetative part was weighed

Figure 9.4: *In vitro* multiplication of Pyrethrum; (a) measurement of the weight of plantlet; (b) and (c) rooting plantlets; (d) development of roots.

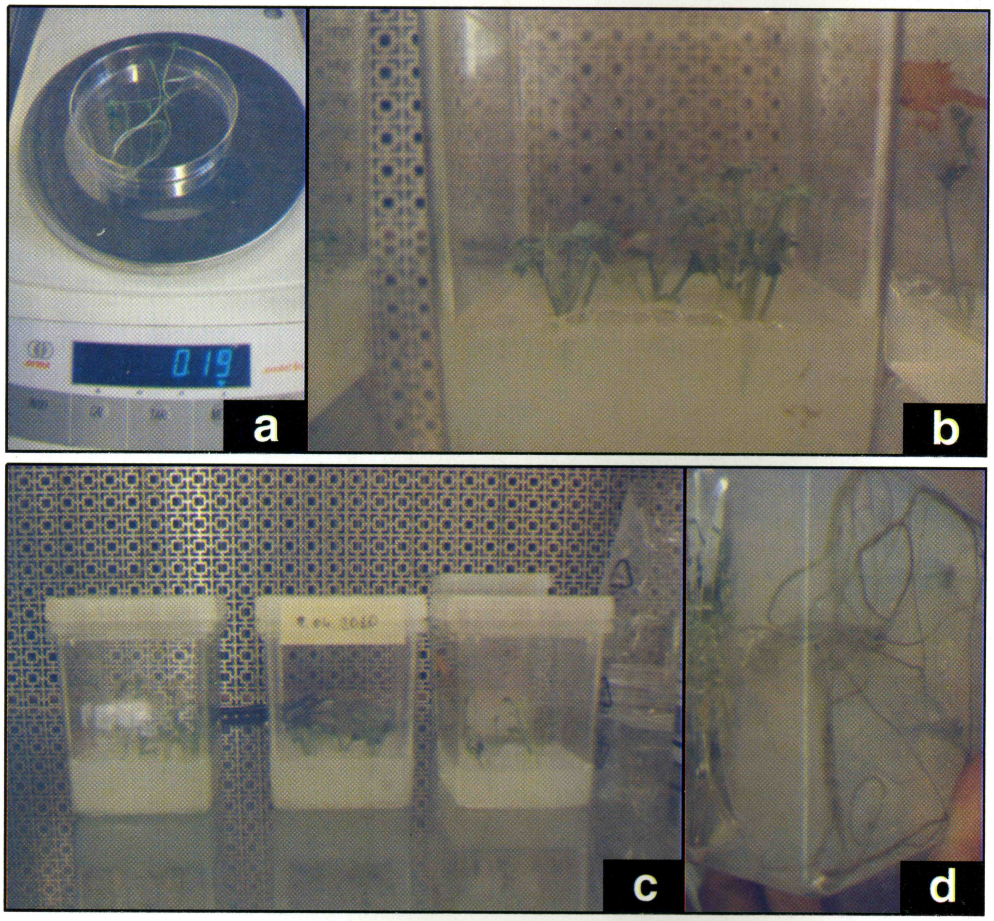


Table 9.5: Number and size of roots in micropropagated pyrethrum shoots (Catalano, 2010). Mean values ± standard deviation (n=10).

Roots mass (g)	*N. of roots**	*Roots length (cm)*
0.06 ± 0.03	7 ± 2	6.35 ± 1.38

* Counting of all adventitious roots.

Ex vitro Acclimatization and Establishment in Field

The acclimatization process of micropropagated plants, previously obtained by means of *in vitro* techniques, requires some time before plants to be ready for transplant in field, and in many cases plants are lost or damaged when transferred to *ex vitro* conditions (greenhouse or field) (Pospíšilová *et al.*, 1999). In pyrethrum, a positive

ex-vitro acclimatization of plantlets was reported by Hedayat *et al.* (2009), who reached a 80 per cent success average; variations may eventually depend upon climatic conditions during the transfer phases (Liu and Gao, 2007). Therefore, in a study about the transfer to *ex vitro* conditions on micropropagated pyrethrum plants, a special attention was also paid in detecting differences on plant establishment caused by environmental variations (Catalano, 2010; Catalano *et al.*, 2011). Hence, the transplant operations were repeated thrice (in April, May and June), using both plants coming from seeds and plants obtained by shoots rooting. Plantlets that had reached a proper size (about 6-7 cm in height) were previously transferred in plastic pots (7 × 7 × 10 cm) filled with a mixture of 60 per cent peat and 50 per cent perlite. At transplant time, a clear polyethylene bag was put on each pot in order to reduce the loss of water due to plant transpiration and therefore to minimize transfer stress; throughout the acclimatization process, the plastic bag was gradually removed. The survived plants were counted 10 days after transplant, and their development was further measured relieving their height values every 7 days. Generally speaking, the plantlets showing the best results after the *ex vitro* acclimatization process were those transplanted in April, with a survival rate of 60 per cent. Many plantlets (75 per cent) that had been transferred in pots in May, otherwise, although showing an initial suitability to the new growth conditions, experienced in the following days evident stress symptoms, in most cases followed by wilting and death. The transplants that were performed in June, only involving plantlets obtained from microshoots, showed an establishment rate of 45.5 per cent.

The plants that achieved the best general vigour conditions were finally transferred in open field (Figures 9.5 and 9.6).

Embriogenic Callus Culture

Callus is an undifferentiated tissue that forms from not specialized cells, multiplying in a disorganized way. It is obtained from tissue explants or cell cultures that, under the stimulus of phytoregulators, are addressing to de-differentiation.

The formation of pyrethrum callus may be obtained starting from leaves (Barthomeuf *et al.*, 1996), petioles (Sarker and Pal, 1991), disk flowers, floral buds and floral stems (Barthomeuf *et al.*, 1996), achenes teguments and receptacle (Zieg *et al.*, 1983). A successful callus formation was achieved by means of binodal segments of stem and by explants from leaves (Catalano, 2010). The Murashige and Skoog (MS) medium, with 3 per cent sucrose and 0.8 per cent agar, was used as a basis, supplemented with two growth phytoregulators for callus induction: 4 ppm β-naphtoxy acetic acid (ANA) (Sigma), and three different concentrations (0, 0.4, 2 and 4 ppm) of 6-benzyl aminopurine (BAP) (Sigma), a cytokinine) (Barthomeuf *et al.*, 1996; Catalano, 2010) The pH of the growth medium was set to 5.8 by means of the addition of 0,1 M of NaOH, thereafter it was autoclaved for sterilization (20 min, 120°C). The explants were put on Petri dishes that were sealed with Parafilm, and transferred in growth chambers (T 22 ± 1 °C, photoperiod 16-h, irradiance 30 µmol $m^{-2}s^{-1}$). Thereafter, every 40 days, all newly-formed callus was separated by the starting explants, and the explants were repeatedly transferred on fresh medium (Figure 9.7).

Figure 9.5: Steps of pyrethrum *ex vitro* acclimatization: transfer to the pot and transplant in open field.

Contd...

Figure 9.5–*Contd...*

Figure 9.6: 2-years old pyrethrum in open field in a semi-arid Mediterranean environment (Experimental farm "Sparacia", Cammarata, AG, Sicily).

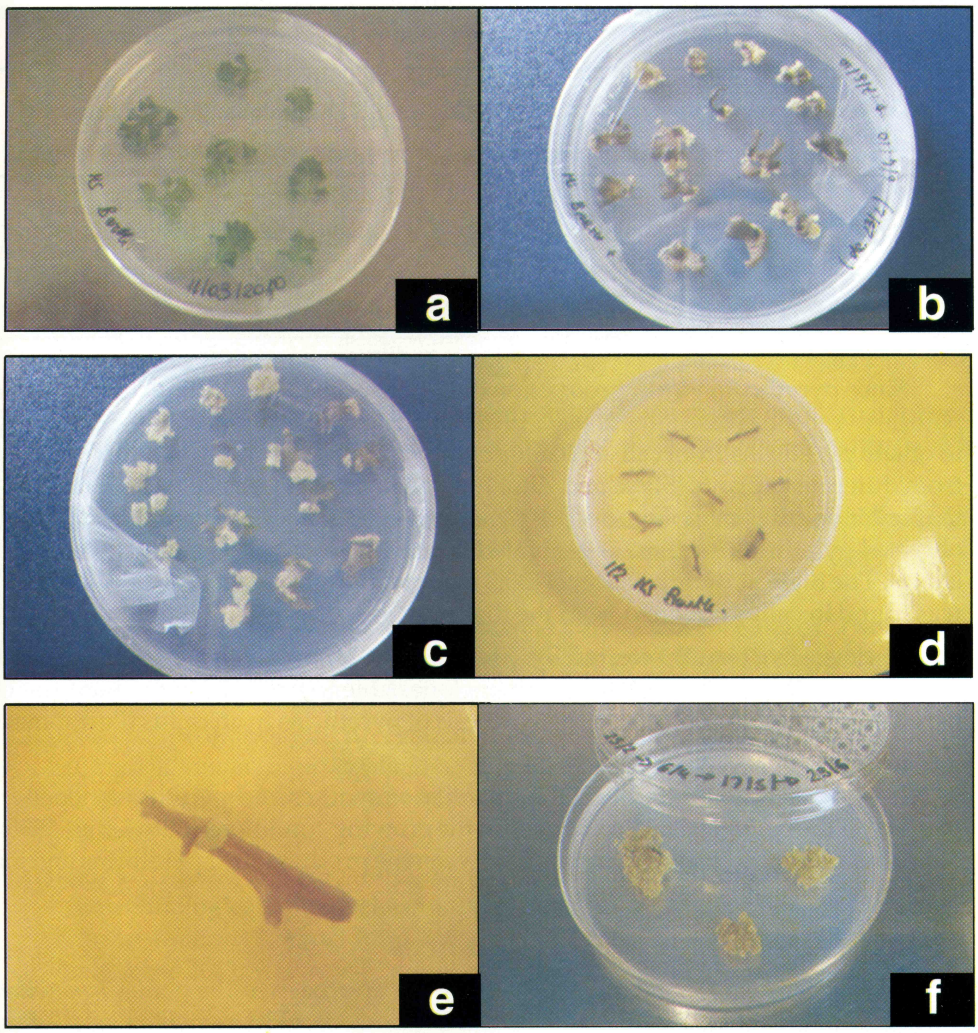


Figure 9.7: Callus formation in pyrethrum; (a) leaves at the beginning of culture; (b) callus from leaves after 40-days (front view); (c) callus from leaves after 40 days (rear view); (d) stem portions at the beginning of culture; (e) callus from the stem; (f) callus masses.

The combined use of an auxine and a cytokinine gave the best outcome, since no callus formation was observed in the medium without BAP (Table 9.6). The highest callus formation (94 per cent) was observed using BAP at 2 ppm di BAP, whereas lower results were obtained with 0.4 ppm (40 per cent) and 4 ppm (5 per cent). Other authors gained good results also using a different mixture of phytoregulators, *i.e.* 2.4-D and BAP (Sarker and Pal, 1991).

Among the parts of plant used for explants, leaves accomplished the best results. In all tested combinations, callus formation had a homogeneous aspect and its colour was varying from whitish to yellow to brown, without taking in any case the colour of chlorophyll (Catalano, 2010).

Table 9.6: Percentage of pyrethrum explants showing callus formation after 40 days culture with different growth media.

Phytoregulators (ppm)		Explant with Callus (per cent)	Stem Portions	Leaves
ANA	BAP			
4	0	**0**	0	0
4	0.4	**40**	14.5	25.5
4	2	**94**	34	60
4	4	**5**	0	5

Since pyrethrum world production does not succeed in fulfilling the request of natural pyrethrins (Barthomeuf *et al.*, 1996), an attractive idea concerns the possibility to obtain pyrethrins from cell and callus cultures, and above all from callus obtained from different explants (Staba and Zito, 1985). Although it was reported (Zito, 1994) that callus cultures cannot be used for pyrethrins production, Barthomeuf *et al.* (1996), obtained pyrethrins from callus generated from disk flowers, flower buds, stems and leaves. The highest pyrethrins level (30.3 mg total pyrethrins/100 g of dry biomass) was found in callus obtained from the disk flowers.

The negative results referred by Zito could probably be related to an improper choice of starting clones: the selection of clones able to produce high quantities of pyrethrins is crucial to optimize the synthesis of pyrethrins from callus (Barthomeuf *et al.*, 1996).

It was moreover stated that the quantity of synthesized pyrethrins depends, besides on the biological factors above, on the growing conditions (Hitmi *et al.*, 1998). No correlation was observed, instead, between the production of pyrethrins and the growth rate of callus (Sarker and Pal, 1991).

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