Micropropagation of *Withania frutescens*: one way to recover reduced or extinct plant populations in the Balearic Islands

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We established a rapid and inexpensive micropropagation protocol for the Solanaceae plant *Withania frutescens* in order to produce a large stock of plant material that can be used for conservation and research programs. This plant has an important ecological value but it has a restricted distribution in the Balearic archipelago (Mediterranean, Spain). Shoot tips and internodia were cultured on Murashige and Skoog (MS) medium supplemented with different auxins and cytokinins. The best medium producing shoot induction, was the control MS with no growth regulators added (94 %). This is very convenient due to the associated advantage of reduction in production costs. Production of roots was best (75 %) on the MS supplemented with indol butiric acid (IBA). The combination of NAA and BAP produced the highest mortality (38 %) of explants. *Withania frutescens* was successfully regenerated *in vitro* from nodal shoot segments excised from young seedlings. Acclimatization and survival of plantlets was very high (100 %). The results of our study will allow producing a stock of plant material in short time and that can be used for conservation and research programs in order to maintain the natural diversity of plants in the small islands of the Balearic archipelago.
Keywords: *micropropagation, tissue culture, nodal explants, rare plants, conservation, islands,* Withania frutescens.

MICROPROPAGACIÓ DE *Withania frutescens*: UNA VIA PER A LA RECUPERACIÓ DE POBLACIONS DE PLANTES DE DISTIBUCIÓ REDUÏDA O EXTINTES DE LES ILLES BALEARS. Es descriu un protocol ràpid i econòmic per a procedir a la micropropagació de la planta micro propagación de la planta Solanacea *Withania frutescens,* amb la intenció de produir un elevat nombre de plantes en un curt període de plaç que puguin esser utilitzades en programes de conservació i d'investigació bàsica i aplicada. *Withania frutescens* és una planta amb un alt valor ecològic però que presenta una distribució molt restringida a l'arxipèlag de les Illes Balears (Mediterrani occidental). La part apical de les tijes i els internodes es varen cultiva en un medi MS (Murashige and Skoog) suplementat amb auxines i citoquinines. El medi que va produir la millor inducció de tijes fou el control MS sense reguladors de creixement afegit (94 %). Això és molt convenient degut a l'avantatja associada amb una elevada producció de plantes a baix cost. La producció d'arrels fou més elevada (75 %) en el medi MS suplementat amb IBA. La combinació de NAA i BAP va produir la mortalitat més elevada (38 %) de plàntules. *Withania frutescens* es regenerà amb molt d'èxit *in vitro* a partir de segments nodals de la tija que es tallaren de plantes joves germinades amb llevors. L'aclimatització i supervivència de les plantes fou molt alta (100 %). Els resultants d'aquest estudi indiquen que és factible produir un stock de plantes en un període curt de temps que

pot esser utilitzat per a la recerca i per establir programs de conservació per al manteniment de la diversitat en les petites illes de l'arxipèlag balear.
Paraules clau: *micropropagació, cultiu de teixits, nodes de plantons, plantes rares, consevació, illes,* Withania frutescens.

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Introduction

Withania frutescens is a woody bush with a restricted distribution in the Mediterranean (Tutin *et al*., 1972). In the Balearic Islands, this plant has an important landscape and ecological value (e.g., berries are consumed by the transaharian migratory birds and by the endemic lizard, *Podarcis lilfordi* (Sáez & Traveset, 1995; Castilla, 1999).

Nevertheless, it has a rare distribution, being present and more abundant in small (< 100 ha) than in big (> 1000 ha) islands (Alcover *et al*., 1993). The factors involved in the presence/ absence or in the relative abundance of this plant in the Balearics are unknown, except for the main island of Cabrera National Park. The population of *W. frutescens* in Cabrera consists of only 8 plants, and it is believed that such reduced population is the result of grazing by the introduced sheeps.

The plant *W. frutescens* can be propagated from seeds. However, because germination is difficult and last up to 5 months even after seed scarification (Castilla, 2000), micropropagation of plants can offer a rapid means of producing a large stock of plants from a minimum of original plant material (e.g. Krogstrup *et al*., 1990; Estades & Medrano, 1990; Moura, 1998; Jain *et al*., 2009; Abraham *et al*., 2010). In this study, we established a rapid micropropagation protocol for *W. frutescens*, in order to produce a large stock of plant material that can be used for conservation and research programs in the Balearics or elsewhere.

Materials and methods

Plant material and decontamination

We used ten plants of 3-5 months and healthy conditions that were germinated in the laboratory (Castilla, 2000). Surface-decontamination of these "in vivo" plants was performed by submersion of leaves and stems in 70 % ethanol (6 sec.). Afterwards, plants were put in commercial bleach 7.5 % (w/v) supplemented with 3 drops/500 ml of Tween 80 for 15 min, and subjected to vacuum for 15 min. Plants were rinsed

	Survival			Mortality	N
Regulator	shoot	root	callus		
None	94	28	0	0	53
24D	0	0	100	0	19
BAP	88	0	0	13	16
NAA	47	0	100	0	17
BAP+NAA	44	0	19	38	16
IBA	95	75	60	0	20
Chi^2	23.3	42.8	70.0	36.1	
Df	5	5	5	5	
P	< 0.001	< 0.001	< 0.001	< 0.001	

Table 1. Percentage of explants that produced shoots, roots or callus, and percentage of mortality of *Withania frutescens* in a medium (MS) with different growth regulators (1 ml/l in all cases) or without them (none). Sample size is indicated (N).
Tabla 1. *Percentatge de plantons que varen produir tijes, arrels o calls, i percentatge de mortalitat de* Withania frutescens *en un medi (MS) amb diferents reguladors de creixement (1 ml/l en tots els casos) o sense ells (cap). S'indica la mida de la mostra (N).*

three times (2, 5, 15 min) with distilled water. Nodal segments from herbaceous stems were cut in pieces of 1-2 cm having at least 2 nodes and 2 leaves each.

Culture conditions

Explants were placed vertically on high-sucrose Murashige and Skoog medium [MSmedium (Duchefa), 30 g/l sucrose (Merck), 4g/l Gelrite (Duchefa)] (Murashige & Skoog, 1962). After approximately two to four weeks, newly developed shoots were subdivided as described above and placed on a low-sucrose [10 g/l sucrose (Merck), 4g/l Gelrite] medium supplemented with 1 mg/l of different growth regulators [6-benzylaminopurine (BAP), naphtalene acetic acid (NAA), 2,4- dichlorophenoxy acetic acid (2,4 D), indole-3-butyric acid (IBA), or the combination of 1 mg/l NAA and 1 mg/l BAP. Cultures on basal medium (MS) were maintained as controls. The pH of the medium was adjusted to 5.7 before adding gelrite, and autoclaved at 120 ºC for 20 min. All cultures were incubated in a growing chamber at 29 ºC and a photoperiod of 16h/8h light/dark cycle and a light intensity of 24 μmol/m²/s.

Acclimatization of plants

Fifteen plants with developed shoots and roots were transferred to soil. We used plastic pots (4x5 cm) containing "sowing and slip soil" (Verta: peatlitter, sand and perlite). Pots were irrigated with water and additional nutrients, and maintained under high (ca 100 %) relative humidity during 2 weeks. After the second week, irrigation and relative humidity was slightly reduced. Acclimatization took place in a growing chamber at 29 ºC and a photoperiod of 16h/8h light/dark cycle and a light intensity of 24 μmol/m²/s. After one month plants were repotted and allowed to growth outside the climate chamber.

Morphological traits of the plantlets were recorded after two months, by measuring with a vernier scale, the shoot length (total length of the shoot up to the tip), shoot diameter determined at basal level of the stem, and the maximum width

Fig. 1. Original seedling of *W. frutescens* from where we collected the shoot tips and internodia for the micropropagation experiment (upper photo), and plants pro-duced *in vitro* for this study (lower foto). All plants were maintained in the Royal Botanic Garden of Madrid (CSIC).
Fig. 1. *Plantons originals de* W. frutescens *d'on es varen obtener les tijes apicals per a iniciar els experiment de micropropagació (foto superior), junt amb les plantes produïdes* in vitro *per a aquest estudi (foto inferior). Totes les plantes es varen mantenir al Jardí Botànic de Madird (CSIC).*

of leaves that were larger than 5 mm. We also counted the number of nodes, the total number of leaves, the number of leaves shorter than 5 mm, and the number of leaves larger than 20 mm. Because the roots produced by *W. frutescens* were rather thin and fragile, we did not measure them.

Results

Most explants developed single shoots by longitudinal growth and upward elongation, thus providing enough material for the proliferation of explants. The percentage of plants that produced shoots, roots or callus, differed significantly among treatments (Table 1). The production of shoots was higher than expected when no growth regulators were added (control) (partial $Chi^2 = 4.7$, d.f. = 1, $P < 0.05$), and was lower than expected in the medium that contained 2,4D (partial $Chi^2 = 13.2$, d.f. = 1, $P < 0.001$). We also observed shoots in the media with other regulators added, however, no significant differences were found between the observed and expected values in all cases (partial Chi^2, $P > 0.05$).

Fig. 2. *W. frutescens* explants being cultured in MS-Control media. Leaves and roots were evident after 4 weeks.
Fig. 2. *Cultiu* in vitro *de* W. frutescens *en un medi control de MS. Les fulles i les arrels s'aprecien després de 4 setmanes.*

Fig. 3. (a-b-c). Explants of *W. frutescens* were successfully acclimatized and grown rapidly in the climatic chamber.
Fig. 3. *(a-b-c). Els plantons de* W. frutescens *produïts* in vitro *es varen aclimatar ràpidament i varen créixer amb èxit dins la cámara climatitzada.*

The production of roots was significantly higher in the medium supplemented with IBA (partial Chi^2 = 27.1, d.f. = 1, $P < 0.001$). No roots were observed in any of the other treatments, except for the control. Explant mortality was only observed in the media BAP and NAA+BAP.

The percentage of acclimatized explants was very high (100 %, n = 15). All plantlets survived the following two months, and 12 of them were measured (Table 2). Regenerated plants from explants showed no morphological differences from those grown naturally from seeds.

Discussion

In vitro micropropagation constitutes a powerful tool in conservation, especially for rare and endangered species, for species with reduced distribution areas or popula-

Fig. 4. *W. frutescens* plants grown *in vitro* and showing flowers after 4 months.
Fig. 4. *Plantes de* W. frutescens *cultivades* in vitro *amb flors després de 4 mesos.*

populations, with scarce seed production, with difficulties for seedling establishment, or with a high medical interest (Krogstrup *et al*., 1990; Estades & Medrano, 1990; Jain *et al*., 2009; Nicasio-Torres *et al*., 2009; Abraham *et al*., 2010).

In this study, even if the sample size used was not large, we established a procedure that is appropriate, inexpensive and fast for the multiplication of *W. frutescens*. Our results showed that the MS medium without growth regulators was the most suitable for shoot induction. This is very convenient due to the associated advantage of reduction in production costs.

Trait	mean ± se (range; mm)
shoot length	44.8 ± 5.9 (10 - 79)
shoot diameter	1.5 ± 0.06 (1.2 - 2.0)
nº nodes	11.5 ± 1.0 (4 - 15)
nº leaves	8.8 ± 0.9 (3 - 15)
nº L < 5	2.0 ± 0.2 (1 - 3)
nº L > 20	1.0 ± 0.9 (0 – 11)
max. L width	24.7 ± 2.2 (11 - 38)
min. L width (≥ 5)	7.6 ± 0.7 (5 – 13)

Table 2. Morphological characteristics of 2 months age *W. frutescens* plantlets (n = 12). It is indicated the shoot length, shoot diameter, number of nodes, total number of leaves, and the number of leaves of different size.

Tabla 2. *Característiques morfològiques de plantons de* W. frutescens *de 2 mesos d'edat (n = 12). S'indica la longitud i diàmetre de la tija, el número de nodes, el número total de fulles i el número de fulles de mida diferent.*

Besides, the use of high level of growth regulators may induce somaclonal variation (Larkin & Scowcroft, 1981).

We also found that the MS medium supplemented with IBA was the best to induce root formation in *Withania frutescens*. Similar results were found elsewhere (in Pierik, 1997).

Also, in *Withania coagulans*, a highly endangered medicinal herb, it was also found a prolific multiplication of axillary buds from the nodal segments when it was cultured on MS medium enriched with BA (Jain *et al*., 2009).

Most explants in our study did not survive the combined treatment of BAP+NAA, suggesting that such combination at concentrations of 1ml/l, must be toxic for this plant.

However, the main objective of this study was not to explore the causes of explant mortality or callus formation, but rather to find an appropriate and successful treatment (among the few tested) to micropropagate *W. frutescens*. It is possible that *W. frutescens* may respond better and faster to other growth regulators or to different concentrations of them. However, we believe that the outcome of our study is useful to micropropagate this plant.

A stock of micropropagated *W. frutescens* plants can be used to conduct short-term reintroduction projects in order to examine the effect of sheep grazing on plantlets survival. However, soil fertility, climate and other factors may have a greater effect on plant species diversity than does grazing (Stohlgren *et al*., 1999). Thus, micropropagated plants can be also used to conduct *ex-situ* experiments (e.g., examine the effect of environmental variation on phenotypic variation and survival of plantlets) that may help to understand the present distribution and abundance of plants in the Balearic Islands. *In situ* experiments

are rather unlikely given the difficulties of access to the islands.

Finally, micropropagated plants, can be used to increase the population size of *W. frutescens* in Cabrera island, if necessary. The convenience of using micropropagated plants in reintroduction programs should be carefully studied to protect genetic diversity (e.g., Iriondo *et al*., 2008). However, when the number of individuals in the population is very low, micropropagation could be justified.

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