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Domestication of micropropagated plants of the spice damiana (*Turnera diffusa*)

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Abstract. Tissue culture propagation was performed on the spice shrub damiana (Turnera diffusa, Willd.) using MS medium (Murashige and Skoog 1962) supplemented with different combinations of the plant growth regulators, 6-benzyl adenine (BA) and indole-3-butyric acid (IBA). Organogenesis of leaf explants from wild plants and explants from propagated cuttings was compared; only the former regenerated complete plants. The highest shooting rate (92%) occurred at a concentration of 10^{-7} M BA plus 10^{-6} M IBA. Regenerated shoots were rooted in MS medium without any plant growth regulators. Foliage productivity of the micropropagated plants under field cultivation was determined yearly over 3 years. The yield increased annually for the first two years. The quantity of essential oils in propagated plants was similar to that of wild plants growing nearby. We propose tissue culture propagation of damiana as a viable means of domestication of this wild plant for semi-arid agriculture in Mexico. Commercial propagation would help to conserve wild populations of damiana that are currently threatened by overharvesting.

Abbreviations
BA - 6-benzyl adenine; IBA - indole-3-butyric acid.

Introduction

The conservation of natural resources, especially wild plant species with commercial value, is an official objective of the current Mexican government. As the market for wild plant products increases, escalating harvests of wild plants endanger the existence of slow growing arid plants.

The wild spice shrub damiana (Turnera diffusa) grows in arid and semi-arid regions of Mexico, the U.S.A., the West Indies and South America (Wiggins 1980). The leaves and stems of this species are used for liqueur flavoring and beverages (Vines 1960), and in traditional medicine as a stimulant, aphrodisiac and diuretic (Arias 1976; Martínez 1979). The damiana eco-type grown in Baja California Sur, Mexico is generally preferred by consumers (unpublished data). At the present time, damiana leaves are heavily harvested from wild plants, causing concern for the plant's survival in the wild. The harvest of wild plants is done for 3 reasons: 1) the seeds can not germinate under laboratory conditions, and seed germination in the wild is rare, thus the first usual step to possible domestication is not available; 2) propagation by cuttings has been unsuccessful on a commercial scale (Sandoval 1982); and, 3) it is easy to collect and sell the foliage of wild damiana.

Previous experiments with damiana tissue culture (Díaz-Rondero and Alcaraz-Meléndez 1987) found that Murashige and Skoog (1962) medium (MS) was better than B5 medium (Gamborg et al. 1976) for callus formation before the initiation of shoot organogenesis and subsequent rooting of microshoots.

The aims of this study were: 1) to domesticate wild damiana plants by means of tissue culture propagation using plant material originating from wild plants and from plants that were propagated by cuttings; 2) evaluate the growth and yield of tissue cultured plants for 3 consecutive years in the field, and 3) compare the essential oil content of wild and tissue culture-propagated plants. Our ecological goal was to provide a method of damiana propagation leading to domestication, thus creating a new potential for rural, arid-zone agriculture in Mexico. It is hoped that an efficient means of damiana domestication will reduce the commercial incentive to harvest wild plants.

Materials and methods

Leaf samples for tissue culture propagation were taken from two types of plants: a) wild plants, and (b) plants propagated from cuttings. The wild plants (30 cm high) were collected from the wilderness area near Todos Santos (23° north latitude and 110° 11′ west longitude), Baja California Sur, Mexico, transplanted into pots (10 cm long X 10 cm wide X 15 cm high) containing sandy soil from their original location, and maintained in the laboratory for one year under natural illumination and temperature ranging from 25-35° C. One year later, they were transplanted to the experimental

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The cuttings were obtained from plants of the same eco-type in the same location. The cuttings were immersed in an aqueous solution of naphthaleneacetic acid (NAA)(2.15 X 10^{-4} M) for 10 min, then dusted with a commercial fungicide (Captan; N-[trichloro methylthio]-4-cyclohexene-1-2-dicarboximide; Rhone Poulenc Agro Co., México). During the rooting period, the cuttings were grown in propagation beds in soil mixture containing sandy soil and medium vermiculite (1:1, v/v) at temperatures ranging from 30-45° C at 67-75 µE m^{-2} s^{-1} light intensity. For experiments using cuttings, 6 leaves were used for each treatment. Likewise, for experiments using wild plants, 6 leaves were used for each treatment.

Young leaves (17-20 mm long) were collected from both types of plants at the same time, washed with 5% filtered commercial detergent (Salvo plus; Procter & Gamble Co.) followed by successive surface disinfection with 95% ethanol (15-20 s) and 10% calcium hypochlorite (10 min), then rinsed 5 times with sterile distilled water. The leaves were cut transversely in the middle, and both segments were placed horizontally with the cut edges touching the medium surface. Two segments were placed in 100 ml glass vessels (55 mm diameter X 72 mm high) containing 20 ml of MS medium and capped with clear autoclavable lids. Six vessels were used for each medium concentration of BA and/or IBA; the experiments were repeated 3 times. The six vessels were supplemented with combinations of 6 different concentrations of IBA (10^{-8} M to 10^{-4} M) and BA (10^{-9} M to 10^{-5} M) for a total of 36 different treatments (Table 1). The medium was prepared with different BA concentrations adjusted to pH 5.7 and mixed with 0.8% agar and 3% sucrose before autoclaving at 121° C for 20 min. IBA (pH 5.7) was sterilized by filtration through a 0.45 µm Millipore filter and added to the sterile medium before solidification.

The explants were incubated under continuous fluorescent light (100 µE m^{-2} sec^{-1}) at 25 ± 2° C for 60 d. After this period, those that developed shoots were transferred monthly over 3 months to fresh MS medium lacking plant growth regulators to develop roots. Due to their relatively large size at the last transfer, the plantlets were moved into glass vessels approximately 750 ml (76 mm diameter X 125 mm high) containing 70 ml of MS medium and sealed with aluminum foil. Finally, after 5 months in the growth chamber, plantlets were transferred to 500 ml pots containing untreated natural sandy soil. The pots were covered with 750 ml glass vessels for one month to acclimatize the plants to the lower relative humidity (40-60%). For further acclimatization, plantlets were placed in the laboratory under indirect daylight (71-109 µE m^{-2}.sec^{-1}) at 25 ± 5° C for 6-8 months. When the plants were 5-8 cm high, they were transplanted 1 m apart in the soil of the experimental field. The plants were irrigated with 33 dm^3 water per m^2 every 8 d for 3 months, and thereafter, every 15 d for the duration of the study. Plants were fertilized once a year in the winter with cow manure at 100 g/plant. Each autumn, the plants were measured and pruned. The pruned leaves and stems were weighed to determine productivity for both fresh weight and dry weight (oven dried at 60° C for 24 h).

Total lipids and essential oils of leaves and stems were extracted using methanol-chloroform (2:1, v/v) for 24 h at 4° C. Then, the extract was filtered, 3 ml of distilled water was added, the mixture was centrifuged, the lower chloroform phase was withdrawn, and the aqueous phase was washed 3 times with 3 ml of chloroform. The resulting solution was dried with N_2, and the total lipids were weighed. The essential oils were obtained by fractionation using alumina column chromatography according to Vázquez-Duhalt and Greppin (1987). Wild plants and micropropagated plants used in the lipid analysis were grown under the same environmental conditions at Baja California Sur and were chosen at random.

The effect of growth regulators on shoot and root formation was statistically analyzed by multifactorial Analysis of Variance (ANOVA) and Least Significant Different Test (LSD). Before analysis, the data were arc-sine transformed because they were of binomial distribution and had to be changed to a normal distribution as required by analysis of variance (ANOVA) at 5% level of significance (Zar 1974). Student's t-test was used to analyze the essential oils data.

Results and discussion

The explants originating from plants that were propagated by cuttings showed no response to any combination of plant growth regulator treatments during the tissue culture procedure. They turned brown and died within 15 d, both times this experiment was performed. This study provides no physiological explanation for this phenomenon. However, it is known that pre-treatments of plant stock can affect the micropropagation properties of its plant parts (Debergh and Zimmerman 1990).

Explants originating from wild plants that were incubated with any combination of the plant growth regulators formed new shoots (Fig. 1), and the growth of callus was minimal.

The best plant growth regulator combination to induce shoots on the explants (92%) was 10^{-6} M IBA plus 10^{-7} M BA. The second best combinations were 10^{-4} M IBA with no BA and 10^{-5} M BA with no IBA, where regeneration was 84% and 87%, respectively (Table 1). However, there was a complex interaction between the two growth regulators, and many treatments were statistically equivalent. Each explant produced had 3 to 5 leafy shoots (Fig. 1).

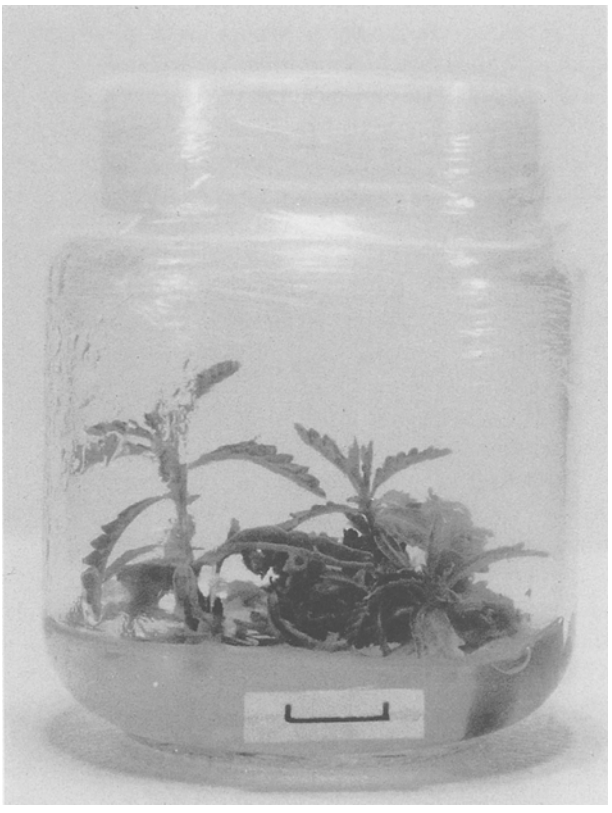

Fig. 1. Plantlets regenerated from damiana leaf explants in MS medium. Scale is 1 cm.

Generally, explant rooting can be promoted in media lacking plant growth regulators. In this study, the most rooting occurred on hormone-free medium in plantlets that had previously been treated with 10^{-8} M IBA (and no BA) or 10^{-5} M BA (and no IBA) for shoot induction (Table 2). In the optimal pre-treatments, 100% of the explants developed roots. Second best rooting was achieved on shoots previously induced with 10^{-6} M IBA plus 10^{-8} M BA, in which 92% of explants developed roots. All other combinations of plant growth regulators in the pre-treatments resulted in lower percentages of rooting (Table 2).

In summary, to produce the greatest number of damiana plantlets through micropropagation, explants should be placed on optimal shoot induction medium (MS + 10^{-6} M IBA + 10^{-7} M BA). Thereafter, they should be transferred to a MS medium lacking plant growth regulators for root development. Under this procedure, rooting was found to take place in 80% of all transferred shoots.

Transplant survival of plantlets from the tissue culture flasks to the pots was 45%, and from pots to the field, survival was 100%. Table 3 shows the growth and the dry weight productivity of the leaves and stems (the commercially viable parts) of micropropagated plants over the 3 consecutive years of this study, and the increased productivity of these plants for the first 2 years. These results can be used to calculate the hypothetical production per hectare under the growing conditions described in our study. The first harvest year would have produced 1200 kg/ha of dry leaves, and the second and third years, 2300 kg/ha.

Table 1. Effect of different concentrations of BA and IBA on shoot production from damiana. Each determination represents 18 replicates expressed as percentage data. [Z]Mean separation by ANOVA and LSD at 5% level with each value transformed to its arcsine.

BA (M)	IBA (M)					
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	35ab[Z]	25a	53abc	50abc	59abc	84bc
10^{-9}	78abc	50abc	61abc	75abc	50abc	63abc
10^{-8}	56abc	67abc	42abc	58abc	69abc	59abc
10^{-7}	75abc	60abc	34abc	92c	67abc	75abc
10^{-6}	74abc	67abc	57abc	73abc	68abc	61abc
10^{-5}	87bc	31ab	26a	55abc	54abc	73abc

The main secondary metabolites extracted from damiana foliage are the essential oils, which are used for flavoring in the liqueur industry or packed for "tea-like" beverages. The quantity of total lipids and essential oils is shown in Table 4. When the lipids and essential oil content between micropropagated and wild plants was compared by student's t-test, there were no significant differences, indicating that micropropagated damiana plants can be useful for the food industry as well. We propose tissue culture propagation as a practical technique for damiana propagation and later domestication. This could make semi-arid lands in Mexico more productive and decrease the probability of over-exploitation and the possible extinction of native damiana populations.

Table 2. Percentage of roots developed in damiana plantlets grown on hormone-free medium following initial cultural steps on different concentrations of BA and IBA. [Z]Mean separation by ANOVA and LSD at 5% level with each value transformed to its arcsine.

BA (M)	IBA (M)					
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
0	75abc[Z]	100c	50abc	90abc	84abc	46abc
10^{-9}	78abc	42a	61abc	74abc	61abc	84abc
10^{-8}	44abc	50abc	75abc	92bc	71abc	54abc
10^{-7}	78abc	72abc	67abc	80abc	86abc	74abc
10^{-6}	48abc	65abc	79abc	67abc	65abc	72abc
10^{-5}	100bc	61abc	56abc	55abc	44ab	44ab

Table 3. Production from 42 damiana plants propagated by tissue culture and planted in the experimental field of the Center for Biological Research of Baja California Sur, located in El Comitan, Baja California Sur, México. The plants were put off every year for three years; results are averages from all the plants sampled. Numbers denoted by a different letter, in each parameter (row) are significantly different at $P \leq 0.05$ by One-way ANOVA and LSD.

	1st year	2nd year	3rd year
Height (cm)	103.7±2.3a	114.9±2.2b	129.8±2.3c
Fresh weight for plant (g)	910.7±73.2a	1266.7±114.2b	1785.4±133.7c
Dry weight for plant (g)	434.2±34.2a	914.1±82.0b	902.2±67.6b
Humidity (%)	45.7	27	44
Leaves: dry weight (g)	159.7±12.6a	346.5±31.1b	347.3±26.0b
Stems: dry weight (g)	274.5±21.6a	579.4±51.0b	539.7±42.0b

Table 4. Lipid content and neutral group lipids from leaves of wild and micropropagated damiana plants (d.w.=dry weight), *SD

	Total lipids (mg/g d.w.)	Essential oils (mg/g d.w.)
Wild plants	77.72±13.03*	32.34±9.41
Micropropagated plants	67.04±15.48	29.33±5.24

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