Scarification of seeds of *Hypericum silenoides* Juss. and its effect on germination

Escarificación de semillas de *Hypericum silenoides* Juss. y su efecto sobre la germinación

Francisca Adriana Mendoza-Urbina, Federico A. Gutiérrez-Miceli, Teresa R. Ayora-Talavera & Reiner Rincón-Rosales*

Departamento de Biotecnología Vegetal, Instituto Tecnológico de Tuxtla Gutiérrez, Tuxtla Gutiérrez, Carretera Panamericana km 1080, C.P. 29050, Chiapas, México.
*reriro61@hotmail.com

ABSTRACT

Hypericum silenoides Juss. is a plant used by traditional medicine by indigenous communities in southeastern Mexico. Under natural conditions their seeds have two major characteristics; these are dormancy and a very poor germination. In this work, different seed treatments, i.e, calcium hypochlorite, sulfuric acid, giberellic acid and hot water, were applied for 30 min each to break dormancy and enhance germination. Immersion of seeds in calcium hypochlorite resulted in 100% germination. The application of 1.5% sulphuric acid yielded 98% germination, the use of giberellic acid at 100 mg l^{-1} resulted in 86%. Immersing the seeds in hot water at 40°C and 50°C also increased germination, but no germination was observed after the 60°C treatment. Seed exposure to calcium hypochlorite considered as the best procedure to break dormancy and promote seed germination.

Keywords: Dormancy, germination, medicinal plant, seed.

RESUMEN

Hypericum silenoides Juss. una planta utilizada en la medicina tradicional por las comunidades indígenas en el sureste de México. En condiciones naturales las semillas tienen dos características principales, que son la latencia y una germinación muy pobre. En este trabajo, fueron aplicados a las semillas diferentes tratamientos por 30 min cada uno, hipoclorito de calcio, ácido sulfúrico, ácido giberélico y agua caliente, para romper la latencia y mejorar la germinación. La inmersión de las semillas en hipoclorito de calcio resulto en un 100% de germinación. La aplicación de acido sulfúrico al 1,5 % produjo 98% de germinación, el uso de ácido giberélico a 100 mg l^{-1} resulto en 86%. La inmersión de las semillas en agua caliente a 40°C y 50°C también aumentó la germinación, pero ninguna germinación se observó después del tratamiento de 60°C. La exposición de la semilla al hipoclorito de calcio fue considerado como el mejor procedimiento para romper la dormancia y promover la germinación de la semilla.

Palabras clave: Latencia, germinación, planta medicinal, semillas.

INTRODUCTION

Hypericum (Hypericaceae) is a large genus of herbs or shrubs which grow in temperate regions of the world (Patocka 2003). The species belonging to this genus have been used as traditional medicinal plants due to their wound-healing (Yazaki & Okuda 1990), bactericide (Ishiguro *et al.* 1998), anti-inflammatory (Dias *et al.* 1998), diuretic and sedative properties (Holz & Ostrowiski 1987) for last two hundred years (Dias *et al.* 2000). There are about 450 species of *Hypericum* in relatively dry temperate zones of the world. Recent studies in different *Hypericum* species revealed the presence of chemicals used in pharmaceutical activity, such as naphthodianthrones, hypericin and pseudohypericin, phloroglucinol, hyperforin and adhyperforin, as well as characteristic xanthones, flavonoids, biflavonoids, tannins and phenolic acids (Barnes *et al*. 2001, Greeson *et al*. 2001). In southeastern Mexico, *Hypericum silenoides* Juss. is an endemic species used by Mayans indigenous communities as a medicinal plant to treat gastrointestinal diseases. For

this reason, interest has emerged for cultivation of this species for pharmaceutical use. Generally, the germination capacity in the case of *H. silenoides* is very poor due to seed dormancy (Macchia *et al*. 1983). Germination is a critical stage in the life cycle of weeds and crop plants and often controls population dynamics, with major practical implications (Keller & Kollmann 1999). Generally, seed germination is restricted by different kind of dormancy in many plant species. Seed dormancy is a block to the completion of germination of an intact viable seed under favourable conditions (Finch-Savage & Leubner-Metzger 2006). In several species of *Hypericum*, dormancy is caused by a chemical inhibitor exudate from the seed capsule (Campbell 1985). For applied uses, dormancy-breaking treatments are required to provide more uniform and rapid seed germination responses. Permeability may be improved by scarifying the seed coat by mechanical means (e.g. clipping, abrasion or immersion in hot water) or chemically with strong oxidative agents (e.g. sulphuric acid or sodium hypochloride) (Abdallah *et al.* 1989). Cirak (2007) reported that seeds of *H. orientale* L. and *H. origanifolium* Willd. treated with 1.5% H_2SO_4 to break dormancy and increased germination significantly. Scarification and In-vitro cultivation offer a viable alternative to achieve germination and obtain genetically uniform plants, which can be the source of major pharmaceutical plant material. The objective of this study was to determine the effect of scarification on seed germination using different treatments such as calcium hypochlorite, sulphuric acid, gibberellic acid and hot water.

MATERIALS AND METHODS

Plant material and collection of seeds

Seeds of *H. silenoides* plants were collected in Chenalho indigenous community fields, Chiapas, Mexico (16°52´35.4˝ N, 92°37´48.5˝ W) where they have been used traditionally as a medicinal plant. The collection place was on a hill, at 1600 m asl, dominated by pine-oak trees and clay soil. At least a whole plant specimen was collected for taxonomic identification so that botanical specimens were deposited in the UNAM (MEXU) National Herbarium. Bulk collections of seeds were randomly selected from the plant population. Seeds were harvested at a similar stage of maturity bases of their colour, cleaned manually and put into paper bags for storing at 4 ºC under dark room conditions until experimental testing (Cirak 2007).

Characterization of seed and viability testing

The morphological characteristics of seeds were examined with the help of a scanning electron microscope and under these conditions the seed size was determined. The absolute seed weight was estimated by counting 1000 seeds in two replications and weighing them on electronic scales with an accuracy of 0.01. Water absorption rate was identified by means of seeds soaking and weighing at certain intervals of time as described (Nedkov 2007). Seed viability was assessed by using 2, 3, 5, triphenyl-tetrazolium chloride (TTC) staining. Seeds were pre-soaked in a solution of 2% calcium hypochlorite for 30 min and washed two times in distilled water, then 10 ml of 1% (w/v) TTC, adjusted to a pH of 6.5 with NaOH, was added and the mixture incubated under dark room conditions at 30°C ±1 for 72 h, and observed under a fotonic microscope. Red stained seeds were considered viable as described (Hartmann & Kester 1983). This proof was replicates three times with 25 seeds in each replicate.

Seeds scarification

Scarification treatments evaluated in this work are shown in Table I. One hundred seeds were treated with 50, 100 and 150 mg l^{-1} GA_3 solutions; 0.5, 1.0 and 1.5 % H_2SO_4 solutions; 1, 2 or 3 % $Ca(ClO)_2$ solutions; 40°, 50° and 60 °C hot water; and with distilled water, each treatment for 30 min. Two additional type of treatments to assess the double effect of combined treatments, using GA_3, H_2SO_4 and $Ca(ClO)_2$ were carried out as follows: In the first test, seeds were immersed in a combined solution made of 150 mg l^{-1} GA_3 and 0.5% H_2SO_4 solution and in the second type of treatment, seeds were immersed in 150 mg l^{-1} GA_3 and then in a 2 % $Ca(ClO)_2$ solution. The seeds soaked in distilled water were used as control. After the application of each treatment, seeds were washed several times until the elimination of the residuals chemical (Cirak 2007). Treated seeds were germinated on 0.8% agar-water plates. Each block of 25 planted seeds was arranged in a completely randomized design with four replicates and placed in plant growth chambers with a photoperiod of 16 h light/8 h darkness at a temperature of 20 °C (Bertelle *et al.* 2004). The percentage of germination, defined as the complete formation of normal seedling plumule (shoot) and primary root, was determined daily between day 6 and day 20. The Kotowski's speed of germination index was calculated 20 days after planting (Kotowski 1926). All statistical analyses were analyzed statistically by ANOVA using SAS software (SAS Institute Inc. 1989), followed by comparison of means by Tukey´s test ($P<0.05$).

RESULTS AND DISCUSSION

Seed characteristics

The seeds of *H. silenoides* are cylindrical shaped with longitudinal mesh wrinkling and brown colour (Fig. 1A). Their average dimensions are: length 1.2 mm and 0.6 mm width. The average weight of 1000 seeds was 19 mg. The maximum amount of water absorbed by seeds was 27 mg and was reached after 72 h of soaking. The viability of *H.*

silenoides seeds was > 89 %, which indicated that the seeds are viable and have the potential to germinate.

Microscopic analysis showed that the seed coat is formed by a double layer that protects the embryo (Fig. 1C) and it isolates him of the external environment, this feature causes dormancy in the seeds. In *Hypericum* species, Corner (1976) mentioned the occurrence of epidermal cells in the testa with brown tannic content and lignified exotegmen and an endotesta formed by a layer of sclerotic cells.

Seed germination

The scarification treatments increased significantly ($P<0.05$) the germination and the dormancy breaking of seeds of *H. silenoides*. It is clear that treatments with 1%, 2% and 3% $Ca(ClO)_2$ for 30 min reported 100% germination and higher values of Kotowski´s speed of germination index 20 days after sowing (Table I) when compared against other treatments. Calcium hypochlorite is an oxidizing agent and acts on the seed cuticle, caused the depolymerisation of lignin in simple phenolic compounds (Buchanan *et al.* 2000) and thus modifying the structure of the sclereid cells that form testa, increasing the permeability and improving germination. *H. silenoides* seeds that were exposed to calcium hypochlorite showed no damage in embryos, because finally showed high percentage of germination and viability. This effect along with the several germination steps can be observed when seeds were analyzed under the electron microscope (see Figs. 1B-D). Vejsadová (2006) found that seeds of *Dactylorhiza incarnata* (L.) Soó*, Dactylorhiza maculata* (L.) Soó and *Liparis loeselii* (L.) Rich. treated with 7.2% calcium hypochlorite for 40 or 50 min significantly increased germination. This allows us to understand the efficiency of calcium hypochlorite to break dormancy in seeds of hard seed coat, such as seed *H. silenoides*.

Table I. Percentage of germination at 6, 10, 16 and 20 days and Kotowski´s speed of germination index 20 days after planting of *Hypericum silenoides* seeds.

Tabla I. Porcentaje de germinación a los 6, 10, 16 y 20 días e índice de velocidad de germinación de Kotowski 20 días después del sembrado de semillas de *Hypericum silenoides.*

Treatment*		Percentage of germination (%)				Speed of germination index after 20 days
		6 days	10 days	16 days	20 days	
GA_3	50 mg L^{-1}	0.0 c**	34.0 cde	65.0 c	70.0 c	6.3
	100 mg L^{-1}	0.0 c	21.0 de	80.0 abc	86.0 abc	6.5
	150 mg L^{-1}	0.0 c	29.0 de	71.0 bc	73.0 bc	6.4
$Ca(ClO)_2$	1%	0.0 c	78.0 ab	100.0 a	100.0 a	7.3
	2%	0.0 c	100.0 a	100.0 a	100.0 a	7.5
	3%	0.0 c	98.0 a	100.0 a	100.0 a	7.3
H_2SO_4	0.5%	11.0 abc	56.0 bcd	83.0 abc	83.0 abc	6.5
	1.0 %	12.0 abc	55.0 bcd	86.0 abc	86.0 abc	6.8
	1.5%	7.0 abc	78.0 ab	98.0 a	98.0 a	7.0
Hot Water	40°C	8.0 abc	31.0 cde	83.0 abc	91.0 abc	6.6
	50°C	3.0 bc	49.0 bcd	94.0 ab	97.0 ab	6.9
	60°C	0.0 c	0.0 e	0.0 d	0.0 d	0
Mixture	(150 mg L^{-1}GA + H_2SO_4 0.5%)	20.0 a	68.0 abc	96.0 a	96.0 ab	7.0
	(150 mg L^{-1}GA + $Ca(ClO)_2$ 2%)	15.0 ab	72.0 ab	88.0 abc	88.0 abc	6.9
Control		0.0 c	33.0 cde	66.0 c	68.0 c	6.5
MSD ($p<0.05$)		3.68	7.99	5.93	6.24	

*All treatments were applied at 30 min. / Todos los tratamientos fueron aplicados durante 30 minutos.

**Mean values of four replicates. The means followed by the same letter are not significantly different ($P<0.05$). / Las medias son promedios de 4 valores. En la misma columna, las medias seguidas con la misma letra no son diferentes estadísticamente ($P<0,05$).

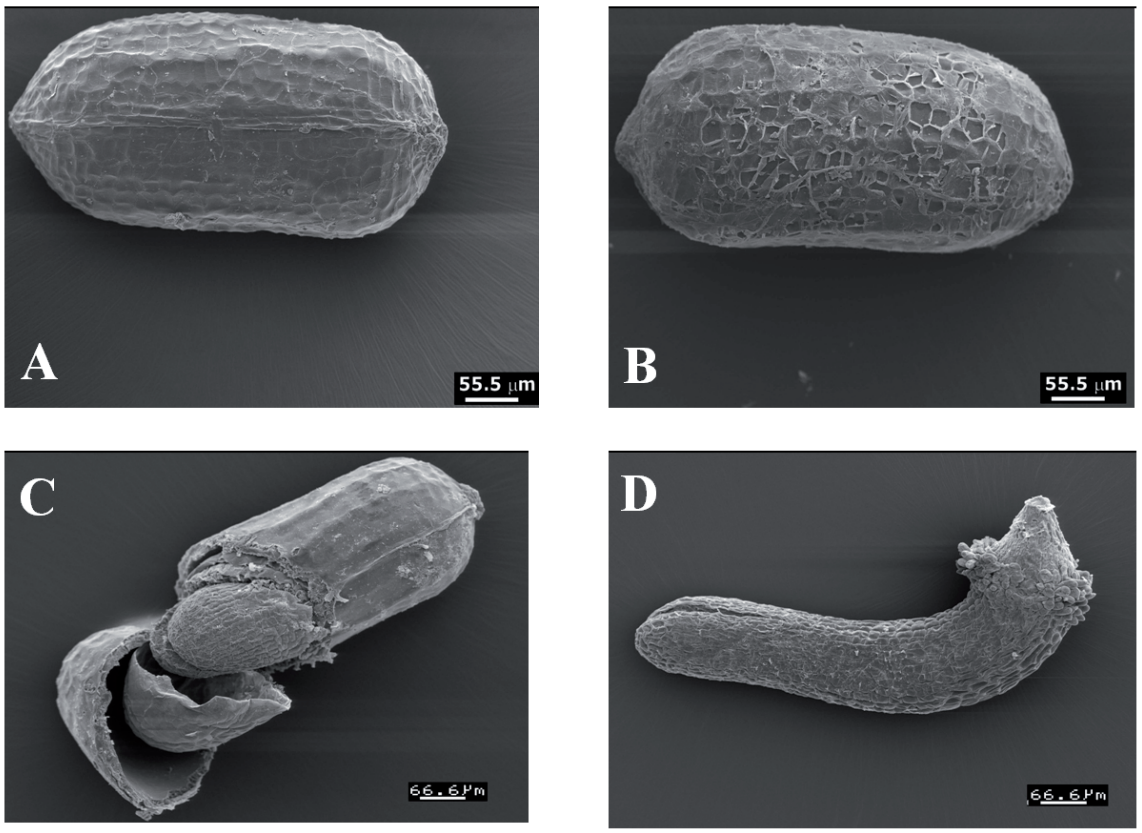

Figure 1. Scanning electron photomicrographs showing the germination process in seeds of *Hypericum silenoides* scarified with calcium hypochlorite. (A) Normal seed, (B) Scarified seed, (C) Germinated seed and showing the layers of coat, (D) Embryo after germination.

Figura 1. Microscopía electrónica de barrido mostrando el proceso de germinación de semillas de *Hypericum silenoides* escarificadas con hipoclorito de calcio. (A) Semilla normal, (B) semilla escarificada, (C) Semilla germinada mostrando las capas de la testa, (D) Embrión después de la germinación.

The application of GA_3 (50, 100 and 150 mg l^{-1}) significantly increased ($P<0.05$) the percentage of seed germination when compared against untreated control seeds (Table I). Cirak (2007) found that application of GA_3 to 100 mg l^{-1} increased germination in *Hypericum perforatum* L. and *Hypericum pruinatum* Boiss. & Balansa. These results are similar to those obtained in this work and can confirm the positive impact of the gibberellins in both breaking dormancy and increasing seed germination. Imbibition stimulates GA_3 secretion from embryo. GA_3 secretion increases synthesis of hydrolytic enzymes located under the aleuron layer. Synthesized enzymes are transported to the endosperm via scutellum and are used for decomposing of stored food to supply the energy required for germination (Debeaujon & Koornneef 2000).

Immersing seeds in 1.5% H_2SO_4 for 30 min significantly increased the percentage of germination and when seeds were treated with 0.5 and 1 % H_2SO_4, reduced the percentage of germination, but still remains higher than those found in untreated control seeds (Table I). Cirak (2007) found that seeds of *Hypericum orientale* treated with H_2SO_4 at concentrations higher than 1 % registered greater germination percentages but when treated with low concentrations found no significant differences. The H_2SO_4 acts by progressive corrosion of the external tegument with a consequent higher permeability to air and water and thus improving the imbibition of seeds and the normal course of the germination process (Muhammad & Amusa 2003).

The scarification of seeds with hot water at 40ºC and 50ºC showed 91% and 97% germination after 20 days of planting, respectively. However, the seeds treated with 60ºC did not germinate (Table I). Immersion of dry seeds in hot water at temperatures up to 50ºC led to their coat rupture allowing water to permeate faster the seed tissues causing physiological changes and the subsequent germination process. Cirak *et al.* (2004) reported that *H. perforatum* seeds treated with hot water at 40ºC for 30 min yield 40% germination and when the temperature was raised at 60ºC, germination decreased 8%. Here it was observed that hot water enhanced germination of hard coated seeds. An increase in water temperature yields a higher permeability of O_2 in the testa (Aydin & Uzun 2001). Cirak (2007)

reported that seeds of *H. perforatum, H. origanifolium* and *H. pruinatum* treated with hot water induce lower levels of germination while in the *H. orientale* was not effective in promoting germination. The negative effects of hot water on the germination of *Hypericum silenoides* seeds were probably due to the combination of both high temperature and time, which may cause damage to the embryo tissue as observed in other *Hypericum* species (Masamba 1994).

The mixture of 150 mg l^{-1} GA_3+ 0.5% H_2SO_4 increased the germination to 96% and 150 mg l^{-1} GA_3+ $Ca(ClO)_2$ obtained 88% when compared to untreated control (Table I). Ai-Rong (2007) reported that GA_3 promotes germination when combined with a scarification treatment. Cirak (2007) reported that seeds treated with 150 mg l^{-1} GA_3+ 0.5 % H_2SO_4 increased the germination of *H. orientale* (50%), *H. origanifolium* (30%) and *H. pruinatum* (55 %).

We found that the *H. silenoides* seeds scarified with calcium hypochlorite appeared to be the best technique to break dormancy and increase germination. This treatment resulted in a highest percentage of germination. The technique is easy to apply and can be used on a lot of seeds at once.

ACKNOWLEDGEMENTS

We thank the plant biotechnology laboratory from the Technological Institute of Tuxtla Gutiérrez (Chiapas, México) for providing facilities for this investigation. The research was funded by DGEST-México under the project SII-2010.

REFERENCES

Abdallah, M.M.F., R.A. Jones & A.S. El-Beltagy. 1989. An efficient method to overcome seed dormancy in Scotch broom (*Cytisus scoparius*). Environmental. Experimental Botany 29: 499-501.

Ai-Rong, L., I. Guan Kai-Yun & R.J. Probert. 2007. Effects of light, scarification and gibberellic acid on seed germination of eight *Pedicularis* species from Yunnan China. Hort Science 42: 1259-1262.

Aydin, I. & F. Uzun. 2001. The effects of some applications on germination rate of Gelemen Clover seeds gathered from natural vegetation in Samsun. Pakistan Journal of Biological Sciences 4: 181-183.

Barnes, J., L.A. Anderson & J.D. Phillipson. 2001. St John´s Wort (*Hypericum perforatum* L.): a review of its chemistry pharmacology and clinical properties. Journal of Pharmacy and Pharmacology 53(5): 583-600.

Bertelle, F.M.L., M.B. Perecin & A.A. Do Lago. 2004. Light, temperature and potassium nitrate in the germination of *Hypericum perforatum* L. and *H. Brasiliense* Choisy seeds. Bragantia 63: 193-199.

Buchanan, B.B., W. Gruissem & R.L. Jones. 2000. Biochemistry and molecular biology of plants. American Society of Plant Physiologist. USA. 849 pp.

Campbell, M.H. 1985. Germination, emergence and seedling growth of *Hypericum perforatum*. Weed Research. 25: 259-266.

Cirak, C. 2007. Seed germination protocols for ex situ conservation of some *Hypericum* species from Turkey. American Journal of Plant Physiology 2: 287-294.

Cirak, C., A.K. Ayan & K. Kevseroglu. 2004. The effects of light and some presoaking treatments on germination rate of St. John's worth (*Hypericum perforatum* L.) Seeds. Pakistan Journal of Biological Sciences 7: 182-186.

Corner, E.J.H. 1976. Clusiaceae. pp 97-103 (1st vol.), pp 92-111(2nd vol.). In. E.J.H. Corner. The seeds of dicotyledons. University Press, Cambridge.

Debeaujon, I. & M. Koornneef. 2000. Gibberellin requirement for *Arabidopsis* seed germination is determined both by testa characteristics and embryonic abscisic acid. Plant Physiology 122: 415-424.

Dias, A.C.P., A. Francisco, T. Barbean, F.M. Ferreria & F. Ferreres. 1998. Unusual flavanoids produced by callus of *Hypericum perforatum*. Phytochemistry 48: 1165-1168.

Dias, A.C.P., R.M. Seabra, P.B. Andrade, F. Ferreres & M. Fernandes-Ferreira. 2000. Xanthone biosynthesis and accumulation in calli and suspended cells of *Hypericum androsaemum*. Plant Science 150: 93-101.

Finch-Savage, W.E. & G. Leubner-Metzger. 2006. Seed dormancy and the control of germination. New Phytologist 171: 501-523.

Greeson, M.J., B. Sanford & A.D. Monti. 2001. St. John's wort (*Hypericum perforatum*), a review of the current pharmacological, toxicological and clinical literature. Psychopharmacology 153: 402-414.

Hartmann, H.T. & D.E. Kester. 1983. Plant propagation. Principles and practices, 4th edn. Engelwood Cliffs, New Jersey: Prentice-Hall.

Holz, J. & E. Ostrowiski. 1987. St John´s worth HPLC analysis of main components and their variability in the populations. Deuts Apothekertz 127: 1227-1230.

Ishiguro, K., N. Nagareya & H. Fukumoto. 1998. A phloroglucinol derivate from cell suspension cultures of *Hypericum perforatum*. Phytochemistry 47: 347-369.

Keller, M. & J. Kollmann. 1999. Effects of seed provenance on germination of herbs for agricultural compensation sites. Agriculture, Ecosystems & Environment 72: 87-99.

Kotowski, F. 1926. Temperature relations to germination of vegetable seed. Proceeding of the American Society for Horticultural Science 23: 176-184.

Macchia, N., A. Benvenuti & L. Angelini. 1983. Germination characteristics of some seeds of medicinal plants. Rastitel'nye-resursy 21: 461-463.

Masamba, C. 1994. Presowing seed treatments on four African *Acacia* species, appropriate technology for use in forestry for rural development. Forest Ecology and Management 64: 105-109.

Muhammad, S. & N.A. Amusa. 2003. Effects of sulfuric acid and hot water treatments on seed germination of tamarind (*Tamarindus indica* L). African Journal Biotechnology 2: 267-279.

Mweetwa, A.M., G.E. Welbaum & D. Tay. 2008. Effects of development, temperature, and calcium hypochlorite treatment on in vitro germinability of *Phalaenopsis* seeds. Scientia Horticulturae 117: 257-262.

Nedkov, N. 2007. Research on the effect of pre-sowing treatment on seed germination of *Hypericum perforatum* L. Bulgarian Journal of Agricultural Sciences 13: 31-37.

Patocka, J. 2003. The chemistry, pharmacology and toxicology of the biologically active constituents of the herb *Hypericum perforatum* L. Journal Applied Biomedicine 1: 61-73.

SAS Institute Inc. 1989. Statistic guide for personal computers. Version 6.04, Ed. SAS Institute, Inc. Cary.

Vejsadová, H. 2006. Factors affecting seed germination and seedling growth of terrestrial orchids cultured In-vitro. Acta Biologica Cracoviensia 48: 109-113.

Yazaki, K. & T. Okuda. 1990. Procyanins a callus and multiple shoots of *Hypericum erectum*. Planta Medica 56: 490-491.

Recibido: 18.10.10
Aceptado: 29.11.10