Gartenbauwissenschaft, **67** (2). S. 50–54, 2002, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Rapid *in vitro* Propagation and Microtuber Production in *Ullucus tuberosus* (Basellaceae)

Schnelle *in vitro* Vermehrung und Mikroknollenbildung bei *Ullucus tuberosus* (Basellaceae)

M. Jordan, A. Amenábar and C. Roveraro
(Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile)

Summary

A rapid *in vitro* propagation system leading to the generation of plantlets and microtubers was developed for *Ullucus tuberosus*. The method consisted of a two phase culture. The first started with nodal sections, each showing 3–4 axillary buds, and was the source of a large number of new shoots (max. 18.3 shoots/flask) which formed within 15 days. Development of new shoots by axillary-bud sprouting and profuse shoot branching took place after 2–4 days in the presence of MS-media supplemented with various growth regulators including NAA, GA_3, BA or Z (Zeatin). The second phase, taking place after 15 days, consisted in subculturing single shoots to promote the production of roots and microtubers. Single shoots, transferred to a liquid (chromatogram paper-bridges) or jellyfied MS-agar medium in various levels of the indicated plant growth regulators, developed roots forming plantlets with 1 or more microtubers within 2 months. The highest tuberization yield was obtained in the presence of NAA (0.3 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.01 mg l^{-1}) resulting in approx. 83 % tuber formation.

Zusammenfassung

Ein zweiphasiges Verfahren zur schnellen *in vitro* Vermehrung von *Ullucus tuberosus* durch Bildung von Pflanzen und Mikroknollen wurde entwickelt. Während der ersten Phase wird, ausgehend von Nodalsegmenten mit jeweils drei bis vier axillären Sprossen, innerhalb von 15 Tagen eine große Anzahl neuer Sprosse (bis zu 18,3 Sprosse pro Kultivierungsgefäß) gebildet. Die Entwicklung von neuen axillären Sprossen und eine intensive Verzweigung der Sprosse setzte dabei nach zwei bis vier Tagen Kultur auf MS Medium mit verschiedenen Wachstumsregulatoren ein. In der zweiten Phase (nach 15 Tagen) wurden einzelne Sprosse auf flüssigem oder festem MS Medium mit verschiedenen Konzentrationen an Wachstumsregulatoren subkultiviert, um die Bildung von Wurzeln und Mikroknollen zu induzieren. Innerhalb von zwei Monaten wurden ein bis drei Mikroknollen pro Spross gebildet. Der größte Ertrag an Mikroknollen (ca. 83 % Knollenbildung) wurde unter Verwendung von 0,3 mg l^{-1} NAA, 0,1 mg l^{-1} BA und 0,1 mg l^{-1} GA_3 erzielt.

Introduction

Much attention has been focused recently on crops that are very important to the culture and subsistence of small farmers in many parts of the world. Concern has been raised about the future security of the genetic resources of different minor crops (IZQUIERDO and DE LA RIVA 2000). In particular, recent attention has been focused on the Andean root and tuber crops (TAPIA 1997; IZQUIERDO and ROCA 1998). *Ullucus tuberosus* (ullucu, ulluco, papa lisa), Fam. Basellaceae, is one of these species: an ancient, little known, but important tuber plant (CÁRDENAS 1989). The plant is native to the highland region of the Andes and represents a valuable food resource traditionally used by the rural population throughout the centuries (MUJICA et al. 2001). At present, this crop represents an interesting alternative for colder sub-tropical regions, as it can be found, still productive, in small plots of the Peruvian and Bolivian highlands up to Venezuela and Colombia. There is a growing agricultural interest to generate alternatives of new nutritious crops, exploring the use of little known resources, as well as a great need to introduce these crops in new areas around the world. Although nutritional values (CASTILLO et al. 1988, TAPIA 1997) and production can be comparable to potato yields (40–50 tons per hectare), plant productivity is usually limited by the presence of viral diseases (ARBIZU and TAPIA 1994). Viral diseases have expanded due to asexual propagation and the lack of desirable selections affected by the different ploidy forms (diploids, triploids and polyploids) of the cultivated ullucus (ROUSI et al. 1986, 1988). The most common viruses described for this species were *Ullucus mosaic potyvirus*, *Ullucus C comovirus*, *Papaya mosaic potexvirus*, and the *Ullucus mild mottle tobamovirus* (BRUNT et al. 1996). As a result, the production of true seeds to remove viruses in this species is limited, since viable seeds are uncommon; true seeds have been produced under controlled circumstances only (ROUSI et al. 1988; NATIONAL RESEARCH COUNCIL 1989; SPERLING and KING 1990). As it has been reported for other tuber plants, the generation of disease free material by means of meristem and shoot-tip culture and chemotherapy, are extremely useful strategies to recover and reproduce valuable productive clones or germplasm now abandoned (STONE 1982; FLETCHER and FLETCHER 2001). This report describes a rapid multiplication technique that allows massive generation of shoots and microtubers of

U. tuberosus, in a 2-step *in vitro* culture. The generation of microtubers from *in vitro* plants makes it possible to solve long-term storage, sorting transport, storage and distribution problems, and promoting an easier and direct use by the farmers.

Material and Methods

Tubers collected in the field were washed and surface disinfected with captan/benlate (0.02 % each) for 30 min under constant agitation, and were planted in the greenhouse for sprouting. After 1 month several sprouts developed and were excised for in vitro culture assays. Shoots were washed with sterile water, disinfected with 3 % Nahypoclorite for 5 min and again washed with sterile water. For *in vitro* shoot proliferation, two polynodal sections of approx. 2.5 cm with 2 to 3 axillary buds (approx. weight 152 mg), were placed horizontally floating on liquid media in 100 ml Erlenmeyer flasks containing 15 ml of an autoclaved liquid MS-medium supplemented with NAA, GA_3, BA and TDZ. The explants were placed horizontally floating in the liquid medium. For microtuber induction, 15 days old nodal sections of about 2.0 cm having 2–3 axillary buds, derived from one of these treatments, were subcultured in media in presence of various combinations of plant growth regulators. These explants were grown in tubes (25 x 130 mm) containing 12.5 ml MS-liquid nutrient medium with 3 % sucrose provided with Whatman Nr 1 paper bridges and in a solidified MS-medium (agarose 0.6 % Sigma, A-0169). Growth regulators closely related to microtuber development described for various species, such as JA (jasmonic acid) and ABA were tested as well as various supplements such as mannitol, proline and thiamine. All explants were maintained in a light regime of 14 hours at 48 $\mu M\, m^{-2}\, s^{-1}$ provided by daylight fluorescent lamps (Philips TLT 40W/54 R.S.) and a temperature of 22±1 °C. Rooted plantlets reaching approx. 5.0 cm were transferred to non sterile conditions using a substrate composed of equal volumes of sand/vermiculite/soil in growth chambers for 1 month acclimatization followed by transfer to the greenhouse.

Results and Discussion

Shoot multiplication

Growth responses observed in this initial phase are summarized in Table 1. The polynodal sections placed horizontally in liquid media in flasks at various hormonal levels showed an extremely rapid growth. Sprouting of axillary buds also occurred in most growth regulator levels, starting within 2–4 days. Total fresh weight increased to a maximum of 2702 mg in the best treatment within 15 days in culture (Fig. 1). Large shoot size and fresh weight variation was found in all the treatments since bud sprouting and subsequent development of new shoots occurred simultaneously along the whole explant. The greatest yield of shoots were obtained in a treatment supplemented with NAA (0.01 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.01 mg l^{-1}). Under these conditions more than 500 new shoots per flask, pink in color, were produced, each averaging 5–8 cm after 2 months (Fig. 2). Generally few roots were

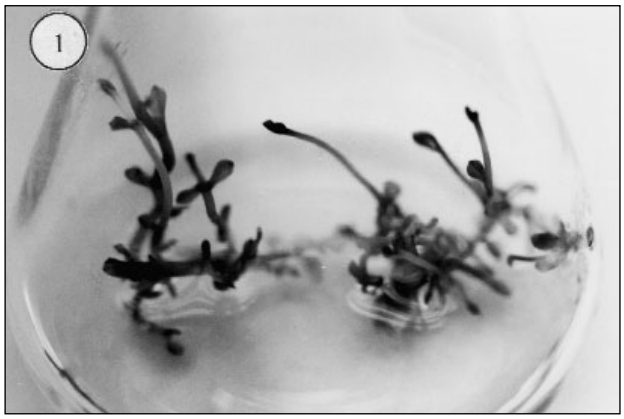


Fig 1. Axillary bud-sprouting and shoot development from nodal sections grown in liquid medium containing NAA, 0.3 mg l^{-1}; BA, 0.1 mg l^{-1}; and GA_3, 0.01 mg l^{-1}, after one week. *Bildung und Entwicklung von axillären Sprossen nach einwöchiger Kultivierung von nodalen Segmenten in flüssigem Nährmedium mit 0,3 mg l^{-1} NES; 0,1 mg l^{-1} BA und 0,01 mg l^{-1} GA_3.*

Table 1. Growth responses of cultured polynodal sections of *Ullucus tuberosus* in the presence of various growth regulators after 15 days [1]
Wachstum von polynodalen Segmenten von Ullucus tuberosus *nach 15 tägiger Kultivierung mit verschiedenen Pflanzenregulatoren.*

Growth Regulators (mg l^{-1})				Total new shoots/ Treatment	Number of shoots/flask X	Shoot length (mm) X±S.D.	Number of roots/flask X	Total fresh weight (mg) X±S.D.	Remarks
NAA	BA	TDZ	GA_3						
0.0	0.0		0.0	18	1.5	3.6±2.86a	0.67	174.4±26.8a	Only elongation, explants lose color
0.01	0.1		0.01	123	18.3	25.2±15.8b	0.67	2702±861c	Profuse growth, pink
0.01	0.5		0.4	65	9.3	20.2±14.6b	0.0	1028±569b	Profuse growth, pink
0.1	0.1		0.04	80	11.7	19.7±12.4b	0.0	1009±370b	Profuse growth, pink
0.3	0.1		0.01	62	8.9	24.5±16.6b	0.0	966±692b	Profuse growth, pink
0.3		1.0	1.0	28	4.0	26.3±17.0b	2.0	1741±767b	New shoots with little growth, thick and pink

[1] Results of 7 Erlenmeyer flasks/treatment. Experiments started with 2 polynodal sections per flask of approx. 3 cm, 3–4 axillary buds and initial fresh weigh of approx. 120 mg each explant. Values followed by different letters are significantly different (Duncan's multiple range test; $P < 0.05$).

formed in this phase. No axillary bud sprouting occurred in media devoid of growth regulators; explants showed a rapid decay with pigment loss when cultured in light or in permanent darkness. The etiolated sections showed a 4 fold size increase due only to internode elongation. Except for treatments started with 2,4-D, shoots did not demonstrate callus formation. Callusing was infrequent and only occurred in pretreatments including 2,4-D (1 mg l^{-1}, for 11 days) at the proximal portions of the explants. Thiamine had a favorable effect in promoting root growth; these roots were finer and longer. However, the shoot/root biomass relation was not different as compared to controls [2.44/1 and 2.57/1, respectively] (Fig. 3c).

Plantlets and Microtubers.

In subculture, shoots responded in different ways, proliferating, tuberizing and/or rooting, according to the plant regulators and addenda employed. In hormone-free medium neither roots nor tuberization occurred while in the presence of various levels of NAA, BA and GA_3, induction of new shoots (Fig. 3b), roots and/or microtuber formation was observed (Table 2; Fig. 3a,c, and d). The best treatment for root and tuber formation was NAA (0.3 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.01 mg l^{-1}) with 100 % plantlets and 83.3 % tuber formation, producing 2 or more microtubers/explant within 2 months. Microtubers were also formed under higher levels of NAA and BA, (2 mg l^{-1}, respectively), although shoot growth was partially inhibited resulting in plantlets with short internodes (Fig. 3a). The new tubers formed at the distal end or along the explant, and rarely at proximal ends. Tubers first appeared red in color turning greenish brown. In some older cultures maintained for 4–6 months, 2–4 microtubers of different size also developed at the time when explants demonstrated rapid decay. The final size of each in vitro forming microtuber varied between 4 and 27 mm in length weighting aprox. 60 to 1000 mg while the biomass donor tissue/microtuber quotient, was observed to be a 1:3–10 proportion in the fresh weight, indicating that very small explants were able to develop microtubers (Fig. 3d). Occasionally, new sprouts developed from the new microtubers (Fig. 4).

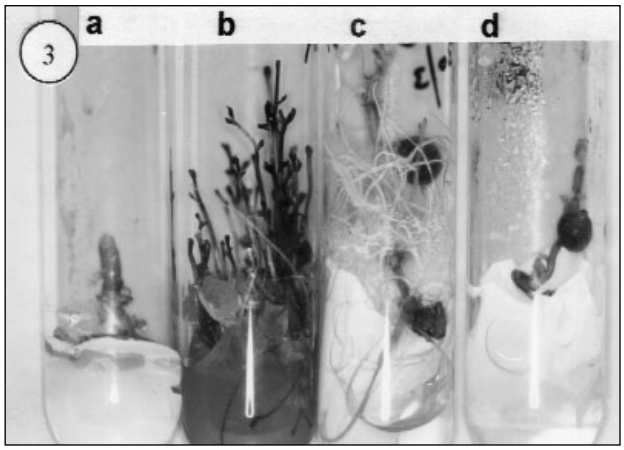


Fig 3. Growth responses induced by different growth regulators on nodal sections in subculture within 2 months (from left to right): a) First stages of tuberization at the distal end of a nodal explant (NAA and BA, 2 mg l^{-1} each). b) Simultaneous development of several shoots in presence of various growth regulators (NAA, 0.02 mg l^{-1}; Z, 2.5 mg l^{-1}; GA_3, 1.0 mg l^{-1}) [no microtuber formation]. c) Shoot development with profuse root proliferation and microtubers in presence of NAA, 0.3 mg l^{-1}; BA 0.1 mg l^{-1}; GA_3, 0.01 mg l^{-1} and supplemented with thiamine, 20 mg l^{-1}. d) Development of a microtuber on a nodal section with little development, pretreated with 2,4-D, 5 mg l^{-1} for 11 days and then subcultured in presence of NAA 0.3 mg l^{-1}, BA, 0.1 mg l^{-1} and GA_3, 0,01 mg l^{-1}.

Ergebnisse der zweimonatigen Subkultivierung von Nodalsegmenten unter Verwendung verschiedener Wachstumsregulatoren. (von links nach rechts):a) Erste Stadien der Tuberisierung am distalen Ende eines nodalen Explantats (2 mg l^{-1} NAA; 2 mg l^{-1} BA). b) Simultane Entwicklung mehrerer Sprosse bei Kultivierung mit verschiedenen Pflanzenregulatoren (0,02 mg l^{-1} NES; 2,5 mg l^{-1} Z; 1.0 mg l^{-1} GA_3) [keine Knollenbildung]. c) Sprossentwicklung mit intensiver Wurzelproliferation und Knollenbildung bei Anwesenheit von 0,3 mg l^{-1} NES; 0,1 mg l^{-1} BA; 0,01 mg l^{-1} GA_3 und 20 mg l^{-1} Thiamin. d) Knollenbildung an einem wenig entwickelten nodalen Segment nach einer elftägigen Vorbehandlung mit 5 mg l^{-1} 2,4-D und anschließender Subkultivierung mit 0,3 mg l^{-1} NES; 0,1 mg l^{-1} BA und 0,01 mg l^{-1} GA_3.

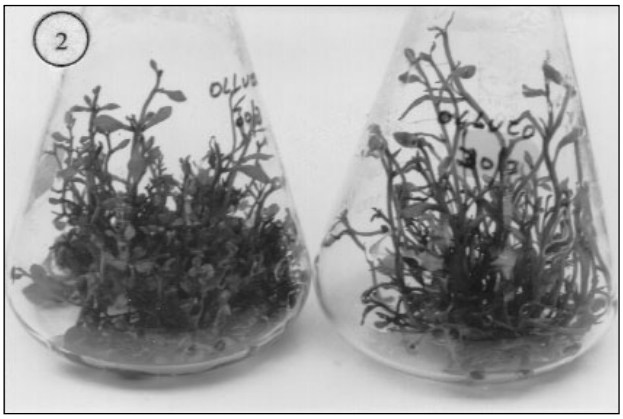


Fig. 2. Profuse growth of new shoots in liquid medium after two months (NAA, 0.3 mg l^{-1}; BA, 0.1 mg l^{-1} and GA_3, 0.01 mg l^{-1}. *Intensives Wachstum neuer Sprosse nach zwei Monaten Kultivierung in flüssigem Nährmedium mit 0,3 mg l^{-1} NES; 0,1 mg l^{-1} BA und 0,01 mg l^{-1} GA_3.*



Fig. 4. Different shapes of microtubers. Some microtubers germinate showing sprouts.

Verschiedene Knollenformen. Einige Mikroknollen keimen und bilden Sprosse.

Table 2. Summarized treatments and morphogenic responses observed in nodal sections of *Ullucus tuberosus* after 2 months in subculture[1].
Zusammenfassung der Versuche und morphologische Veränderung nodaler Segmente von Ullucus tuberosus *nach zweimonatiger Subkultivierung*

Pre-treatment 2,4-D, 11 days ($mg\,l^{-1}$)	Plant Regulators & Addenda				Plantlet/ Treatm. %	Microtuber formation %[2]	Remarks
	NAA	BA	Z	GA_3			
	($mg\,l^{-1}$)						
–	–	–	–	–	0	0	Internode elongation only. Explants lose color
–	0.02	–	2.5	1.0	0	0	Many new shoots, no roots.
–	0.3	0.5	–	0.1	0	16.7	Axill. bud sprouting, no roots.
–	0.3	0.1	–	0.01	100	83.3	Best plant regulator level for microtuber formation.
1.0	0.3	0.1	–	0.01	100	33.3	Callus at proximal end
2.0	0.3	0.1	–	0.01	100	33.3	Callus at proximal end.
5.0	0.3	0.1	–	0.01	20.8	25.0	Explants lose color. Callus at proximal end.
– JA	0.3 (0.1,	0.1 1.0,	– 10.0)	0.01	8.3-4.2-0.0 respectively	0	Growth and microtuber inhibition
– ABA	0.3 (5.0,	0.1 10.0,	– 30.0)	0.01	0	0	Growth inhibition.
–	0.3 Thiamine 20	0.1	–	0.01	100	54.2	Profuse root proliferation.
–	0.3 Mannitol (2 %), Sucrose (1 %)	0.1	–	0.01	0	0	Growth inhibition, explants lose color
–	0.3 Mannitol (2 %), Sucrose (1 %), Proline ($10\,mg\,l^{-1}$)	0.1	–	0.01	0	0	Growth inhibition, explants lose color
–	2.0	2.0	–	–	12.5	29.2	Shoots compressed (agar medium)[3]
–	2.0	2.0	–	2.0	4.2	79.2	Bud sprouting, few roots.

[1] Started with 24 tubes per culture from shoots grown in presence of NAA ($0.01\,mg\,l^{-1}$), BA ($0.1\,mg\,l^{-1}$) and GA_3 ($0.01\,mg\,l^{-1}$). [2] Tuberization was observed in some cultures after a month. [3] With exception of this treatment, all experiments carried out in liquid media.

Microtubers could be maintained in the tubes over 4–5 months, tolerating desiccation. Pretreatments with 2,4-D reduced tuber formation while the addition of JA at concentrations of 0.1, 1.0 and $10.0\,mg\,l^{-1}$or ABA ($5\,mg\,l^{-1}$, $10\,mg\,l^{-1}$and $30\,mg\,l^{-1}$) totally suppressed tuberization. Inhibition occurred when JA and ABA were used alone or in combination with the growth regulators that promoted tuberization. Strong inhibition was also observed in the presence of mannitol and proline; within 30 days explants became pale experiencing chlorophyll decay and losing their original red color. These JA and ABA effects differ from other results found in various tuberous species, such *as Dioscorea batatas* (KODA et al. 1991), *D. alata* (BAZABAKANA et al. 1999); *Solanum tuberosum* (PELACHO and MINGO-CASTEL 1991) and *Ipomoea batatas* (JARRETT and GAWEL 1991) where a strong tuberization induced by both compounds was observed. Various reports have also established that high levels of cytokinins promote tuberization (PALMER and SMITH 1969; MANTELL and HUGO 1989). This is probably the case with U. tuberosus, since cytokinins (in combination with NAA, $0.1\,mg\,l^{-1}$ or higher), appeared to be necessary to promote sustained growth following microtuber formation in this species. On the other hand, while gibberellins have been reported to inhibit tuberization in potato (MENZEL 1980; VREUGDENHIL and STRUIK 1989), all the levels of GA_3 tested allowed tuber formation. In the presence of a high GA_3 concentration ($2.0\,mg\,l^{-1}$), tuber formation was approx. 79 % as compared with the same treatment devoid of GA_3 (approx. 29 %). The promoting effect of GA_3 levels on microtuber formation is probably a specific response of the species to this hormone. Current results, in combination with other techniques, including meristem culture and antiviral agents (STONE 1982; FLETCHER and FLETCHER 2001), could be relevant for the rescue of valuable germplasm as well as the recovery of diseased clones of the many distinct types of this species remaining in the Andean highlands (NATIONAL RESEARCH COUNCIL 1989).

The author thanks Ms. A. Goreux for revision of the manuscript.

Literature

ARBIZU, C. and M. TAPIA 1994: In: J. E. HERNANDO-BERMEJO and J. LEÓN (eds.). Neglected Crops: 1492 from a Different Perspective. Plant Production and Protection Series N° **26**. FAO, Rome, Italy, 149–163.

BAZABAKANA, R; M. L. FAUCONNIER; B. DIALLO, J. P. DUPONT and J. HOMES 1999: Control of *Dioscorea alata* microtuber dormancy and germination by jasmonic acid. Plant Growth Regulation **27,** 113–117.

BRUNT, A. A., K. CRABTREE, M. J. DALLWITZ, A. J. GIBBS, L. WATSON and E. J. ZURCHER (eds.) 1996: Plant Viruses On-line: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. URL http:// biology.anu.edu.au/ Groups/MES/vide/.

CÁRDENAS, M. 1989: Manual de Plantas Económicas de Bolivia. Ed. Los Amigos del Libro, La Paz.

CASTILLO, R; C. NIETO and E. PERALTA 1988: El germoplasma de cultivos andinos en Ecuador. VI Congreso Internacional sobre Cultivos Andinos, Memoires. INIAP, Quito, Ecuador.

FLETCHER, P. J. and J. D FLETCHER 2001: In vitro virus elimination in three andean root crops: oca (*Oxalis tuberosa*), ulluco; (*Ullucus tuberosus*), and arracacha (*Arracacia xanthorrhiza*). New Z.J. Crop & Hort. Sci. **29,**23–27.

IZQUIERDO, J. and W. ROCA 1998: Under-utilized Andean food crops: status and prospects of plant biotechnology for the conservation and sustainable agricultural use of genetic resources. Acta Horticulturae **457,** 157–172.

IZQUIERDO, J. and G. A DE LA RIVA 2000: Plant biotechnology and food security in Latin America and the Caribbean. Electronic Journal of Biotechnology 3 N°1. URL http://www.ejb.org/content/ vol3/issue1/ full/1/index.html .

JARRETT, R. L. and N. GAWEL 1991: Abscisic acid-induced growth inhibition of sweet potato (*Ipomoea batatas*) *in vitro*. Plant Cell Tiss. Organ Cult. **24,** 13–18.

KODA, Y and Y. KIKUTA 1991: Possible involvement of jasmonic acid in tuberization of yam plants. Plant Cell Physiol. **32,** 629–633.

MANTELL, S. H. and S. A. HUGO 1989: Effects of photoperiod, mineral medium strength, inorganic ammonium, sucrose and cytokinin on root, shoot and microtuber development in shoot cultures of *Dioscorea alata* L. *and D. bulbifera* L. yams. Plant Cell Tiss. Organ Cult.**16,** 23–37.

MENZEL, C. M. 1980: Tuberization in potato *Solanum tuberosum* cultivar Sevago at high temperatures: responses to gibberellin and growth inhibitors. Ann. Bot. **46,** 259–266.

MUJICA, A. S., S. E. JACOBSEN and J. IZQUIERDO 2001: Potencial de los Cultivos andinos, actualmente subutilizados por la biotecnología. IV Encuentro Latinoamericano en Biotecnologia Vegetal, RED-BIO-2001, 4–8 de Junio, Goiania, Brasil. Libro de Resumos, 59–60.

NATIONAL RESEARCH COUNCIL 1989: Last Crops of the Incas: Little-known Plants of the Andes with Promise for Worldwide Cultivation. National Academy Press, Washington.

PALMER, C. E. and O. E. SMITH 1969: Cytokinins and tuber initiation in the potato *Solanum tuberosum* L. Nature **221,** 279–280.

PELACHO, A. M. and A. M. MINGO-CASTEL 1991: Jasmonic acid induces tuberization of potato stolons cultured in vitro. Plant Physiology **97,** 1253–1255.

ROUSI, A.; J. SALO, R. KALLIOLA, P. JOKELA, L. PIETILA and M. YLI-REKOLA 1986: Variation pattern in ullucus (*Ullucus tuberosus*, Basellaceae), a supposedly asexual Andean tuber crop. Acta Horticulturae **182,** 145–152.

ROUSI, A.; M. YLI-REKOLA, P. JOKELA, R. KALLIOLA, L. PIETILA and J. SALO 1988: The fruit of *Ullucus* (Basellaceae) an old enigma. Taxon **37,** 71–75.

SPERLING, C. R. and S. R. KING 1990: Andean tuber crops: worldwide potential,. In. J. JANICK and J. E. SIMON (eds.). Advances in new crops. Timber Press, Portland, OR, 428–435.

STONE, O. M. 1982: The elimination of four viruses from *Ullucus tuberosus* by meristem-tip culture and chemotherapy. Ann. Appl. Biol. **101,** 79–83.

TAPIA, M. E. 1997: Cultivos andinos subexplotados y su aporte a la alimentación. FAO, Santiago.

VREUGDENHIL, D. and P.C. STRUIK 1989: An integrated view of the hormonal regulation of tuber formation in potato. Physiol. Plant. **75,** 525–531.

Eingereicht: 15.12.01/ 05.02.02

Anschrift der Verfasser: Jordan, M. Amenábar, A. and Roveraro, C., Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. E-mail: mjordan@genes.bio.puc.cl