INFLUENCE OF NITROGEN SUPPLY ON MICROPROPAGATION AND SUBSEQUENT MICROTUBERIZATION OF FOUR POTATO CULTIVARS

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Abstract

A medium containing low amounts of nitrogen (19-23 meq.l^{-1}) produced optimum results in micropropagation as revealed by the number of nodes, internode length, chlorophyll content, and leaf area of four potato cvs. belonging each to four different maturity groups. Decreasing amounts of nitrogen also increased chlorophyll content in all four cultivars tested. The NH_4^+ concentration did not have an effect on micropropagation for low nitrogen supplies.

In all cvs., except Baraka, there was a "carry over" effect of the nitrogen content in the micropropagation medium onto subsequent tuberization, the lower nitrogen (23 meq.l^{-1}) advancing tuber initiation. Microtuberization of cv. Jaerla was earlier in darkness than under short days regardless of the propagation medium used.

Resumen

Un medio con bajo contenido en nitrógeno (19-23 meq.l^{-1}) permitió obtener unos resultados óptimos en la micropropagación que fue evaluada midiendo el número de nudos, la longitud de entrenudos, el contenido en clorofila y el área foliar de 4 cultivares de papa pertenecientes a 4 grupos de madurez distintos. La disminución de nitrógeno en el medio producía un aumento en el contenido en clorofila en los 4 cultivares ensayados. La concentración de $NH4^+$ no modificó la micropropagación para bajos aportes de nitrógeno.

En todos los cultivares, excepto Baraka, hubo un efecto retardado del contenido de nitrógeno en el medio de micropropagación sobre la posterior tuberización, de manera que un contenido bajo de nitrógeno (23 meq.l^{-1}) anticipó la tuberización. La microtuberización del cv. Jaerla fue más precoz en oscuridad que en días cortos, independientemente del medio de propagación utilizado.

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Introduction

Potato tissue culture is central to the development of cellular and molecular techniques. Rapid multiplication of genetically engineered material by micropropagation facilitates the availability of new transgenic plants in agricultural production.

Media composition is rarely adjusted to the particular physiological process under study but rather is "borrowed" from a standard medium selected for other purposes, such as MS (Murashige, T. and F. Skoog, 1962) which was developed for tobacco pith callus. As Pierik suggested (Pierik, R.L.M., 1987), nutrient levels in MS medium are often higher than required for optimal plant development.

Growth and multiplication rate of single-node potato explants in micropropagation depends on the cultivar (Caligari, P.D.S. *et al.* and Evans, N.E., 1993). Major factors limiting the rates of micropropagation were short overall height of the plantlets and crowding of the nodes (Miller, *et al.*, 1985). In order to find an improved standard protocol allowing the micropropagation of recalcitrant cultivars (those showing low multiplication rates, short internodes, or poor leaf development), we selected four cultivars belonging to four different maturity groups. In previous experiments cvs. Jaerla and Baraka showed slower vegetative growth than cvs. Spunta and Turia (Fig. 1).

A high micropropagation response, which was fairly uniform over cultivars, was obtained (Caligari, *et al.*, 1989) at 20 C and 3% sucrose. It was also reported (Amirouche, *et al.*, 1985) that MS medium was, in general, superior to Knop's medium when micropropagation was initiated from tuber sprouts.

Some evidence indicates that nitrogen levels during micropropagation, in addition to affecting growth directly, may also produce other delayed effects. Thus, nitrogen levels greatly influenced shoot morphology (Evans, N.E., 1993) and the development of potato stem sections during the micropropagation stage depended on the previous culture medium on which the shoot tips were initially grown (Amézqueta, J.M., *et al.*, 1989).

The purpose of this research was to study the effect of nitrogen content and NH_4^+ concentration on micropropagation and subsequent in vitro tuberization of four potato cultivars. Special attention was paid to parameters such as number of nodes and internode length in recalcitrant cultivars.

Materials and Methods

Plant Material

Single-node stem sections of potato, *Solanum tuberosum* L. cvs. Jaerla (early), Spunta (middle-early), Turia (middle-late) and Baraka (late), cultured in vitro, were used throughout the experiments. Tuber sprouting and culture initiation were performed as previously described (Mingo-Castel, *et al.*, 1991), with the following modifications. Tubers were surface-sterilized with 1% (v/v) NaOCl plus 0.1% (v/v) Tween-20 for 5 min, and then rinsed three times with sterile tap

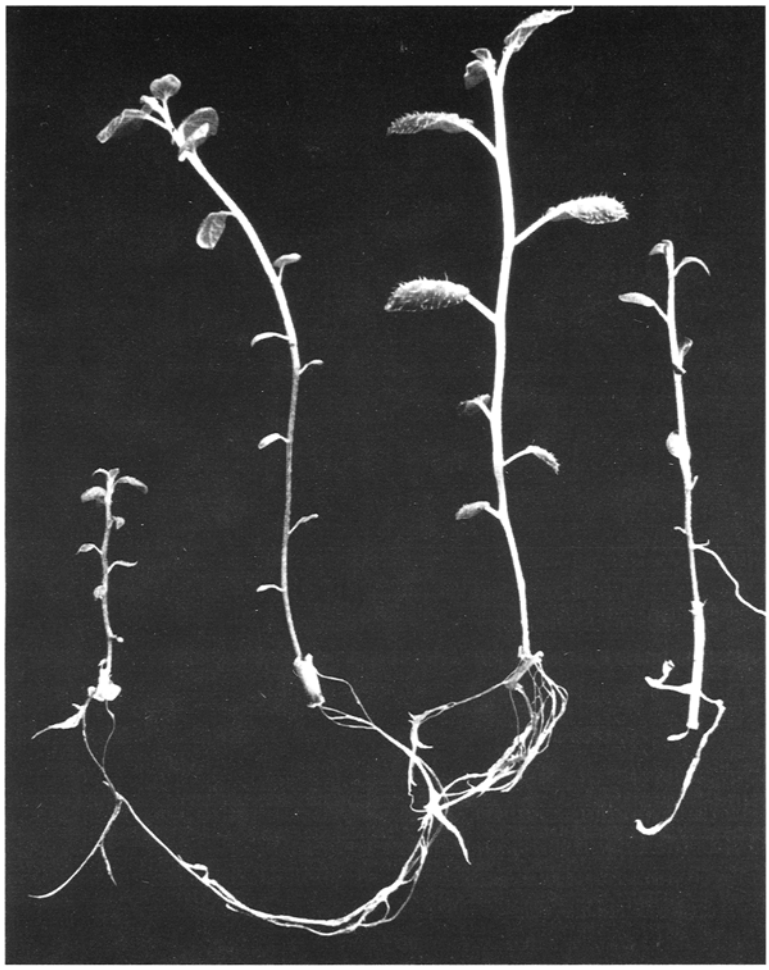

FIG. 1. Morphology during micropropagation of the 4 potato cvs. tested. From left to right: Jaerla, Spunta, Turia and Baraka.

water. Tuber plugs, 2 cm in diameter and 2.5 cm thick, each containing a single bud, were individually excised from tubers with the aid of a cork borer. Plugs were incubated, in plastic trays containing moist vermiculite, in the dark and sprayed with $Ca(NO_3)_2.4H_2O$ 27.7 mM to avoid apical necrosis. Single-node sprout sections were aseptically cultured in autoclaved Murashige & Skoog's medium containing 2% (w/v) sucrose and 0.8% (w/v) Difco Bacto-agar. Cultures were maintained at 20 C under 16 h photoperiod and 70 $\mu moles.m^{-2}.s^{-1}$ photon flux using Philips TLD/84 fluorescent lamps. Henceforth, three subcultures of leafless single-node shoot sections were made under above conditions to collect enough material for the micropropagation study.

Micropropagation

To assess the effect of nitrogen supply on micropropagation, Murashige and Skoog's mineral salts (Murashige and Skoog, 1962) were used with the exception of NH_4NO_3. Different concentrations of NH_4NO_3 were added to modify the nitrogen supply and the NH_4^+ concentration, without affecting any other ion concentration. Media "N1", "N2", "N3" and "N4" contained 357, 60, (as in MS), 23 and 19.2 meq.l^{-1} nitrogen. All media contained 2% sucrose and 0.8% Difco Bacto-agar. The nitrogen composition of culture media is shown in Table 1.

Cultures were grown at 20 C under 16 h photoperiod and a photon flux of 70 µmoles.$m^{-2}.s^{-1}$. Twenty-four explants were cultured for each treatment and cultivar. The experiment was performed twice.

After four weeks in culture the leafless single-node shoot sections had developed in a shoot in which the following parameters were measured: number of viable nodes, internode length, leaf area and chlorophyll content (mg chlorophyll per g fresh weight) of the third fully expanded leaf from the shoot apex. Leaves were removed and chlorophyll measurements were carried out using a Minolta chlorophyll meter SPAD-502. Leaf area was measured with a LI-COR portable area meter, model LI-3000A.

Microtuberization

Leafless single-node shoot sections of all cvs. were cultured on MS-derived N2 or N3 media (Table 1) in the micropropagation stage. Four weeks later new lateral branches developed, and they were used as a source of whole single-node shoot sections for subsequent microtuberization experiments.

The microtuberization medium was composed of MS mineral salts (60 meq.l^{-1} nitrogen), 6% sucrose, 11.6 µM kinetin and 0.8% Difco Bacto-agar. A negative control included the same components except kinetin.

Cultures were grown both under 8 h photoperiod and 70 µmoles.$m^{-2}.s^{-1}$ photon flux or in darkness. All of them were kept at 20 C. Twenty-four explants were cultured for each treatment and cultivar. The experiment was performed twice. After 4 and 8 weeks the tuberization percentage (number of explants

TABLE 1.—*Nitrogen composition of culture media.*

Medium	N meq.l^{-1}	KNO_3 meq.l^{-1}	NH_4NO_3 meq.l^{-1}	NH_4^+ meq.l^{-1}	NO_3^- meq.l^{-1}
N1	357	18.8	169	169	188
N2(*)	60	18.8	20.6	20.6	39.4
N3	23	18.8	2.09	2.09	20.9
N4	19.2	18.8	0.19	0.19	19

*is MS medium.

with at least one microtuber over the total number of explants cultured) was measured.

Statistics

Multiple comparisons between treatment means were made using Fisher LSD test at the 5% level. The variables "number of nodes", "internode length" and "tuberization percentage" were transformed using the equations $y=x^{1/2}$, $y=Ln(x+1)$ and y=arcsenx, respectively.

Results

Micropropagation

A significantly lower number of nodes for every cultivar was obtained in medium N1 with the highest nitrogen content (Fig. 2). There were no significant differences in the number of nodes for cvs. Jaerla and Spunta with the three media N2, N3 and N4. However Turia reached optimum values for N2 and N3 treatments and Baraka for N3. For every cultivar tested, internode length increased as nitrogen content decreased, eventually levelling off for N3 in all cultivars except Spunta in which the highest value was obtained for N4 (Fig. 3). The longest internodes, almost two cm long, were obtained in medium N4 for cv. Spunta.

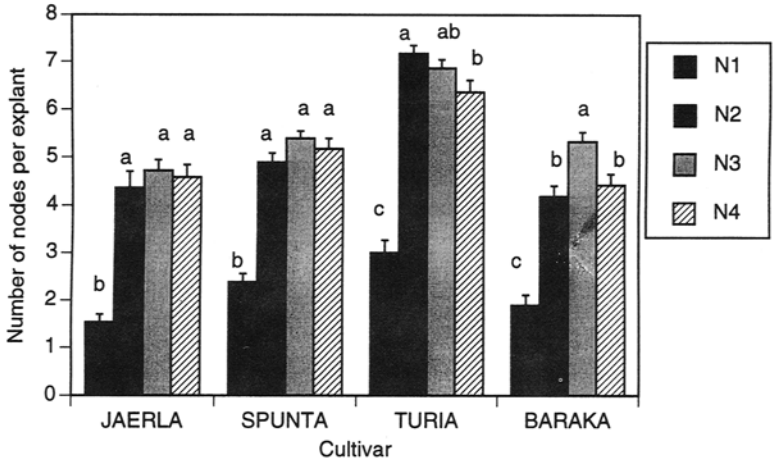

FIG. 2. Number of nodes per explant obtained with 4 different culture media for 4 cvs. Values are averages from 24 explants, and the experiment was performed twice. Different letters, within each cv., indicate statistically different values at p=0.05 using Fisher LSD test. Bars at the top of each column represent standard errors.

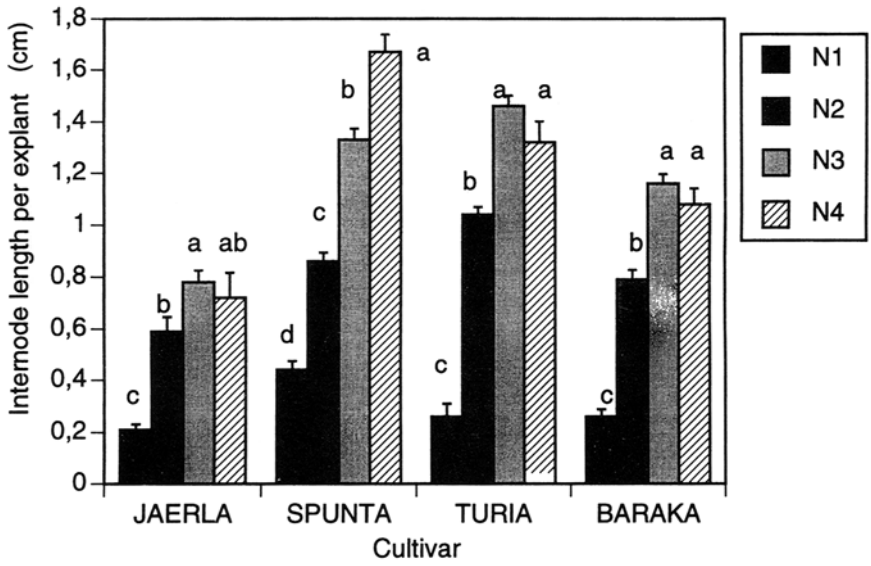

FIG. 3. Explant internode length obtained with 4 different culture media for 4 cvs. Values are averages from 24 explants, and the experiment was performed twice. Different letters, within each cv., indicate statistically different values at p=0.05 using Fisher LSD test. Bars at the top of each column represent standard errors.

The smallest leaf area appeared with the highest nitrogen content in all cvs. except Baraka in which leaf growth was not affected by nitrogen supply (Fig. 4). Media N2, N3 and N4 allowed good leaf growth in Spunta and Turia. The greatest leaf development in Jaerla was recorded with medium N3.

A decrease in nitrogen supply resulted in higher chlorophyll contents for all cultivars (Fig. 5) The two lowest supplies N3, N4, produced the highest chlorophyll content in Spunta and Turia. No significant differences were observed between N2, N3 and N4 in Jaerla and Baraka.

Microtuberization

A significantly higher tuberization in cv. Jaerla was obtained for explants micropropagated on medium N3 than on N2 after 4 weeks, both in darkness or under short days (Fig. 6). After eight weeks no differences were observed between treatments, regardless of photoperiodic regime or nitrogen supply in the previous subculture.

A similar trend was found in cvs. Spunta and Turia, but differences were not statistically significant. Tuberization in cv. Baraka was not affected by nitrogen supply.

Cultivar Jaerla showed in darkness a significantly higher tuberization than in short days after 4 weeks for both nitrogen levels (Fig. 6).

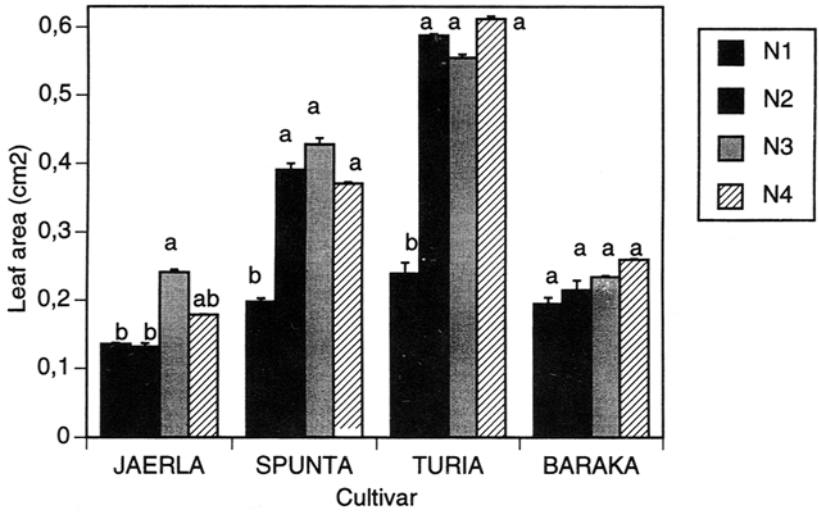

FIG. 4. Leaf area in micropropagated plantlets obtained with 4 different culture media for 4 cvs. Values are averages from 24 explants, and the experiment was performed twice. Different letters, within each cv., indicate statistically different values at p=0.05 using Fisher LSD test. Bars at the top of each column represent standard errors.

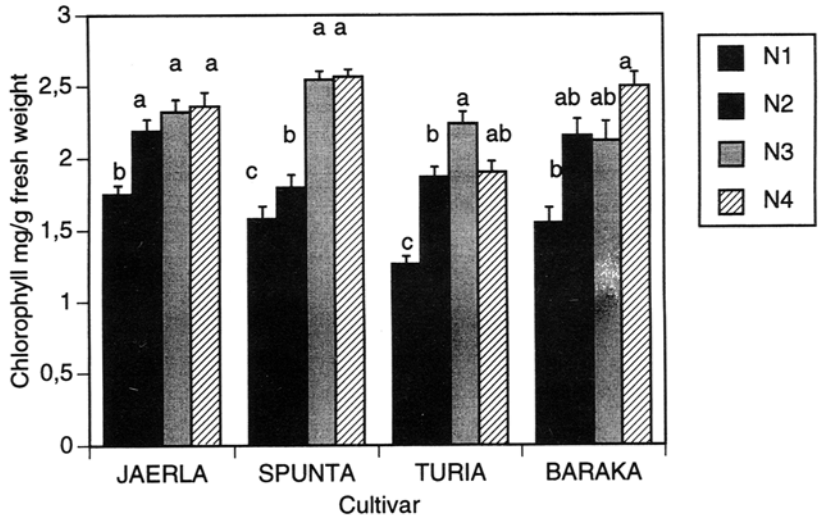

FIG. 5. Leaf chlorophyll content of micropropagated plantlets obtained with 4 different culture media for 4 cvs. Values are averages from 24 explants, and the experiment was performed twice. Different letters, within each cv., indicate statistically different values at p=0.05 using Fisher LSD test. Bars at the top of each column represent standard errors.

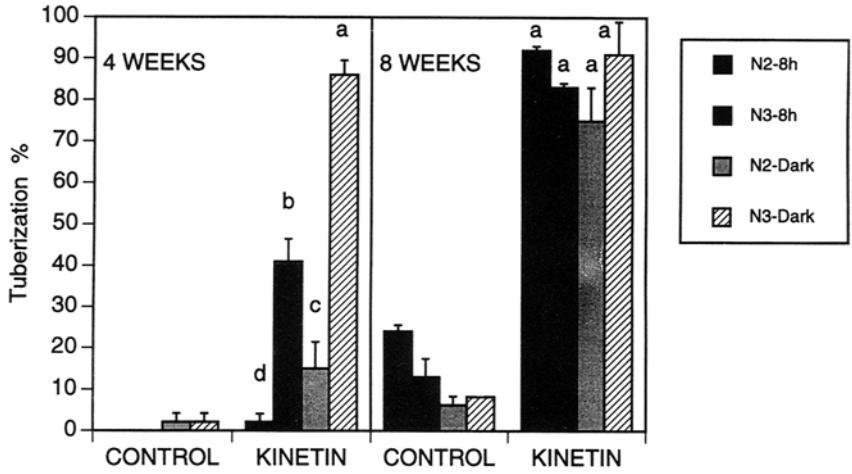

FIG. 6. Kinetin-induced tuberization of potato explants, cv. Jaerla, under 8h photoperiod or in darkness. Explants were obtained after micropropagation in two media (N2 or N3) having different nitrogen supplies. Values are averages from 24 explants, and the experiment was performed twice. Different letters, within each measuring time (4 or 8 weeks), indicate statistically different values at p=0.05 using Fisher LSD test. Bars at the top of each column represent standard errors.

Discussion

The growth parameters measured (number of nodes, internode length, leaf area and chlorophyll content) revealed that the nitrogen level of the medium significantly influenced potato micropropagation.

A nitrogen supply higher than that in MS medium (Murashige and Skoog, 1962) (357 meq.l^{-1} compared with 60 meq.l^{-1}) had a negative effect on every parameter measured, especially the number of nodes (less than half that of other treatments) and internode length. Short lateral outgrowths, which were useless for micropropagation, were observed for the highest nitrogen concentration in all cultivars, except Jaerla.

Best growth occurred in every cultivar at nitrogen levels (23 or 19 meq.l^{-1}), being lower than that of MS medium. Evans (1993) found that the chlorophyll content in response to nitrogen regime was highly dependent on genotype. Thus, decreasing nitrogen from 60 to 20 meq.l^{-1} either did not have a significant effect (in 5 cvs.), or changed chlorophyll content (in 4 cvs.). We found, working with a wider range of nitrogen levels, that a decrease in nitrogen increased chlorophyll content in all 4 cvs. tested.

Concerning the slow growing cultivars (Jaerla and Baraka), the best results for most of the parameters measured were obtained with low nitrogen supplies (23 and 19 meq.l^{-1}). Only the number of nodes in Baraka, was significantly

higher for 23 meq.l^{-1} than for 19 meq.l^{-1}. This is in agreement with the results obtained by Evans (1993) with different potato genotypes. Similar responses were found in cultivars that showed good development in standard MS medium (Spunta and Turia). Again low nitrogen supplies (23 and 19 meq.l^{-1}) produced the best results. Moreover, the lowest nitrogen level was significantly better for the number of nodes and internode length in Spunta. Therefore, an efficient way to improve potato micropropagation may be to use a nitrogen supply lower than that in MS medium.

By comparing the results obtained in media N3 and N4 we may infer the relevance of the NH_4^+concentration on shoot development, since both media have nearly the same nitrogen and NO_3^- levels but differ in NH_4^+ concentration by a factor of ten. There was not a clear difference in overall micropropagation between both treatments in cv. Jaerla. A greater number of nodes was obtained in Turia and Baraka for 23 meq.l^{-1} compared to 19 meq.l^{-1}, but the opposite was true for internode length in Spunta. Therefore, it seems that the amount of NH_4^+ does not have an effect on potato micropropagation, at least at low nitrogen supplies.

Micropropagation of cv. Jaerla in a low nitrogen medium resulted in a significantly earlier tuberization than micropropagation in MS medium (Fig. 6). Nitrogen withdrawal has been generally found to favor tuber initiation (Ewing, *et al.*, 1992). In hydroponic systems (Krauss, 1985) such an effect may depend on the level of irradiance (Ewing, *et al.*, 1992). A very low nitrogen level (5 meq.l^{-1}) resulted in the greatest number of tubers when small in vitro plantlets were transfered to darkness and allowed a better tuberization even in the absence of cytokinins (Teixeira, *et al.*, 1991).

Microtuberization of cv. Jaerla was earlier in darkness than under short days regardless of the propagation media used. Progressive shortening of the light regime with time tends to increase microtuberization. Thus, a change from long to short day conditions promoted earlier tuber formation (Garner, *et al.*, 1989) or tuber diameter in different cvs. (Seabrook, *et al.*, 1993), an effect that might be expected from the growth of the whole plant. Also changes from long days to darkness (Levy, *et al.*, 1993 and Slimmon, *et al.*, 1989) and from short days to darkness (Dobranszki, *et al.*, 1993) promoted or accelerated in vitro tuberization in different systems.

This study suggests that the nitrogen supply of MS medium is too high for optimal stem elongation of four potato cultivars with different growth patterns in MS medium. Lowering the nitrogen level in the culture media is a convenient method to improve micropropagation rates and facilitate handling of in vitro cultured germplasm.

Acknowledgment

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