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Growth of embryogenic sweet orange callus on media varying in the ratio of nitrate to ammonium nitrogen

Randall P. Niedz
United States Department of Agriculture, Agricultural Research Service, U.S. Horticultural Research Laboratory, 2120 Camden Road, Orlando, Florida 32803, USA

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Abstract

Embryogenic callus from *Citrus sinensis* (L.) Osbeck cv. Hamlin was cultured for 28 days on 20 media arranged in a 5 × 2 × 2 factorial varying in the ratio of nitrate to ammonium nitrogen, total inorganic nitrogen, and benzyladenine. Fresh weight increase of callus and final medium pH were significantly affected by total inorganic nitrogen and the ratio of nitrate to ammonium. The nitrate to ammonium ratio accounted for 55% of the variation in the fresh weight increase of the callus and 93% of the variation in the final medium pH. Varying the NO_3^-:NH_4^+ ratio provided adequate pH control.

Abbreviation: BA – benzyladenine

Introduction

Two ionic forms of inorganic nitrogen (N) are available for uptake by plants from the soil - nitrate (NO_3^-) and ammonium (NH_4^+). Both N forms are generally included in tissue culture media; however, many of the early tissue culture media (Gautheret 1939; White 1939; Hildebrandt et al. 1946; Miller & Skoog 1953) used only NO_3^-. One significant contribution of the popular Murashige and Skoog (MS) medium (Murashige & Skoog 1962) was the demonstration that NH_4^+ could be beneficial for plant tissue growth in culture. In addition to the ionic form of N, the NO_3^- to NH_4^+ ratio can affect a tissue's growth and response in culture. For example, Grimes & Hodges (1990) reported that the morphology of regenerated indica rice shoots was strongly affected by the NO_3^-:NH_4^+ ratio.

In *Citrus*, protoplast-derived plants are readily obtained from embryogenic callus and suspension cultures (Kobayashi et al. 1985; Niedz 1993), and have been successfully used to generate somatic hybrids (Grosser & Gmitter 1990; Tusa et al. 1990; Ohgawara et al. 1991), cybrids (Vardi et al. 1987), and transgenic plants (Vardi et al. 1990; Hidaka et al. 1990; Moore et al. 1992). Physiological studies and genetic manipulations require cultured cells or tissues that are healthy and rapidly growing. In this paper the influence of the medium NO_3^-:NH_4^+ ratio on embryogenic *Citrus* callus growth, and the NO_3^-:NH_4^+ ratio's influence on medium pH is presented.

Materials and methods

An embryogenic callus line (H89) from *Citrus sinensis* (L.) Osbeck 'Hamlin', initiated as described by Kobayashi et al. (1985), was grown for 3 years in 100 × 15 mm polystyrene culture dishes sealed with Parafilm (American National Can, Greenwich, CT, USA) containing MS medium supplemented with 10 mg l^{-1} thiamine·HCl and pyridoxine·HCl, 5 mg l^{-1} nicotinic acid, 5% sucrose, 45 μM BA and 0.8% (w/v) bacteriological agar; the pH was adjusted to 5.8 prior to autoclaving. H89 was maintained by subculturing onto

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fresh medium every 28 days and placed in a growth cabinet set at 27 °C with a 4-h photoperiod (15–20 $\mu molm^{-2}s^{-1}$, cool-white fluorescent lamps).

For all experiments callus was transferred 28 days after the last subculture to the treatment media. The first experiment tested 20 media variations differing in inorganic N levels, NO_3^-:NH_4^+, and the presence of BA. The basal medium was a modified MS medium containing 10 mg l^{-1} thiamine·HCl and pyridoxine·HCl, 5 mg l^{-1} nicotinic acid, 5% sucrose, no growth regulators, 0.8% bacteriological agar, and a pH adjusted to 5.8 prior to autoclaving for 15 min at 103 kPa and 121 °C. Two inorganic N levels—30 and 60 mM; five NO_3^-:NH_4^+ ratios—0:100, 25:75, 50:50, 75:25, and 100:0; and two BA levels—0 and 45 μM were examined. The NO_3^-:NH_4^+ ratios were generated using KNO_3, NH_4NO_3, and $(NH_4)_2SO_4$. For example, when 60 mM inorganic nitrogen was supplied, the following salt combinations were used: 0:100 ratio, 30 mM $(NH_4)_2SO_4$; 25:75 ratio, 15 mM NH_4NO_3 and 15 mM $(NH_4)_2SO_4$; 50:50 ratio, 30 mM NH_4NO_3; 75:25 ratio, 15 mM NH_4NO_3 and 30 mM KNO_3; 100:0 ratio, 60 mM KNO_3. For 30 mM inorganic nitrogen treatments, these values were reduced by one-half. Further optimization experiments used NO_3^-:NH_4^+ ratios between 50:50 and 100:0. Treatments were arranged in a completely randomized design and replicated seven times with inorganic N levels, NO_3^-:NH_4^+, and BA as independent variables. Each replication consisted of approximately 100 mg of callus transferred to a 60 × 15 mm polystyrene petri dish containing 6 ml of medium. Dishes were sealed with Parafilm. The fresh weight of each callus was recorded at the beginning of the culture period.

To induce somatic embryogenesis in H89, the callus was subcultured onto the same medium used for maintainance, but 2% glycerol (Ben-Hayyim & Neumann 1983) was substituted for 5% sucrose.

For all experiments data were collected after 28 days in culture. The percent increase in callus fresh weight and the final medium pH were recorded. Medium pH was determined by removing the agar from a dish, mixing it with 8 ml of distilled H_2O for 5 min, and recording the pH. An analysis of variance (ANOVA) and regression analysis were performed for the percent increase in callus fresh weight and final pH of media. The percentage fresh weight gain data were transformed to logs, since it violated the assumption of a homogeneity of variances as determined by Bartlett's test (Little & Hills 1978). The contribution of a source of variation to total variation was calculated by dividing the sums of squares (SS) by total SS and multiplying the resulting product by 100.

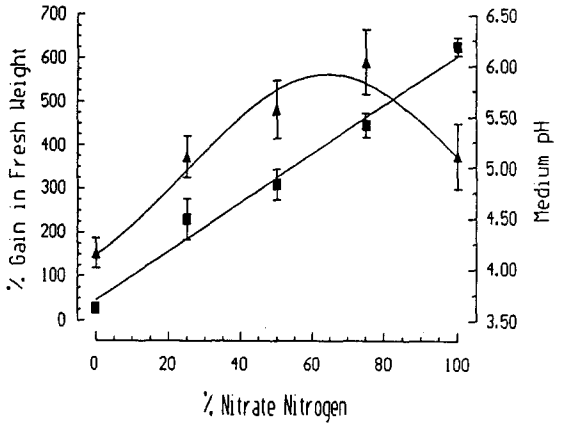

Fig. 1. Percent gain in fresh weight and final medium pH vs % NO_3^-. ▲ $Y = 10^{(2.1720+0.0180(X)-0.00014(X)^2)}$ where Y = % gain in fresh weight and X = % NO_3^-. ■ Y = 3.6957 + 0.0244(X) where Y = medium pH; X = %NO_3^-. Embryogenic callus (about 150 mg) from *C. sinensis* line H89 was transferred onto media containing 5 different proportions of nitrate. The medium pH was determined 28 days later. Mean values ± SE, n=7.

Results and discussion

Increase in fresh weight

The 0:100 NO_3^-:NH_4^+ ratio treatments all showed little growth compared to the other N ratio treatments (Table 1 and Fig. 1). The full model (i.e. NO_3^-:NH_4^+ ratio, N level, BA, and their interactive effects) was highly significant ($p < 1\%$, F test) and accounted for 75% of the observed variation (Table 2). All sources of variation were significant except the 2-way interaction N level × BA. The contribution of each variable to explainable variation (i.e. the 75% fraction) was 74% for the NO_3^-:NH_4^+ ratio, 11% for N level, < 1% for BA, and the remaining 14% was divided among the 2-way and 3-way interactions.

The NO_3^-:NH_4^+ ratio accounted for most of the explainable variation. Fitting a curve to the gain in fresh weight over the range of NO_3^-:NH_4^+ ratios examined revealed a highly significant fit for a quadratic model – log % fresh weight gain = $2.172 + 0.018(\%NO_3^-) - 0.00014(\%NO_3^-)^2$ (Table 3 and Fig. 1). This quadratic model accounted for 54% of the total variation while the linear model, log % fresh weight gain = $2.347 + 0.004(\%NO_3^-)$, accounted for 26%.

Table 1. Percentage weight gain of H89 callus and the final medium pH after 28 days growth on 20 media combinations. Each value is the mean of seven replications.

BA (45 μM)	N level (mM)	N Ratio (NO_3^-:NH_4^+)	Weight gain	Medium pH
+	60	0:100	258	3.71
+	60	25:75	390	5.00
+	60	50:50	398	5.14
+	60	75:25	824	5.66
+	60	100:0	428	6.41
+	30	25:75	491	4.37
+	30	50:50	599	4.80
+	30	75:25	563	5.40
+	30	100:0	583	6.07
–	60	0:100	104	3.64
–	60	25:75	387	4.56
–	60	50:50	362	4.96
–	60	75:25	501	5.63
–	60	100:0	228	6.26
–	30	0:100	122	3.54
–	30	25:75	258	4.03
–	30	50:50	617	4.42
–	30	75:25	525	5.13
–	30	100:0	337	6.03

Basal medium was a modified MS medium containing (mg l^{-1}) 10 thiamine and pyridoxine HCl, 5 nicotinic acid, 5% sucrose, no growth regulators, 0.8% agar, and the pH adjusted to 5.8 prior to autoclaving.

Table 2. ANOVA of log % gain in fresh weight and final medium pH of orange embryogenic cell line H89 after 28 days in culture on media varying in NO_3^-:NH_4^+ ratio, N level, and presence of BA.

		% Gain in fresh weight[a]	Medium pH
Source	df	SS	SS
N ratio	4	5.95 **	106.07 **
N level	1	0.91 **	1.32 **
BA	1	0.03 ns	4.54 **
N ratio × N level	4	0.28 **	0.48 **
N ratio × BA	4	0.52 **	0.79 **
N level × BA	1	0.03 ns	0.01 ns
N ratio × N level × BA	4	0.32 **	0.27 **
Error	120	2.72	0.68
Total	139	10.76	114.16

** $p < 0.01$
ns = not significant
[a] ANOVA on log transformed data

Lack of fit sums of squares was not significant, indicating the proposed quadratic model was reasonable. Slightly better multiple regression models could have been developed, but their biological usefulness would have been questionable considering the small contri-

Table 3. Regression of log % fresh weight gain and medium pH onto % NO_3^-, and of log % fresh weight gain onto medium pH.

	Log % weight gain (y) % NO_3^- (x)[1]		Medium pH (y) % NO_3^- (x)[2]		Log % weight gain (y) Medium pH(x)[3]	
Source	df	SS	df	SS	df	SS
Total, corrected	139	10.78	139	114.15	139	10.78
Regression	2	5.83**	1	104.48**	2	5.60**
Residual	137	4.95	138	9.67	137	5.18
r^2		0.54		0.92		0.52

[1] Based on the quadratic model, $Y = 2.1720 + 0.0180X - 0.00014X^2$
[2] Based on the linear model, $Y = 3.6957 + 0.0244X$
[3] Based on the quadratic model, $Y = -2.6193 + 2.0126X - 0.1892X^2$
** $p < 0.001$

bution to total variation of the other variables (Table 2).

An experiment with total inorganic N set at 60 mM and composed of 50, 60, 66, 70, 80, 90, or 100% NO_3^- was set up to further optimize the NO_3^-:NH_4^+ ratio (data not shown). MS medium has 60 mM inorganic N of which 66% is NO_3^-. It was not possible to resolve an optimum level between 50% and 90% NO_3^-. However, there was a reduction in growth on the 100% NO_3^- medium, and the callus grown on the 90% NO_3^- medium was almost white whereas callus grown with lower levels of NO_3^- had a slight yellowish cast to it.

pH

The full model (i.e. NO_3^-:NH_4^+ ratio, N level, BA, and their interactive effects) was highly significant (Table 2, $p < 0.1\%$, F-test) and accounted for 99% of the total variation. All sources of variation were significant except the 2-way interaction, N level × BA. The contribution of each variable to total variation was 93% for NO_3^-:NH_4^+ ratio, 1.2% for N level, 4% for BA, and the remaining 1.3% contributed by the 2-way and 3-way interactions. Although N level, BA, and the interactions were statistically significant, they had little practical significance. With a sums of squares (SS) of 106.07 out of a total SS of 114.16, the NO_3^-:NH_4^+ ratio accounted for the majority of the variation.

Regression analysis of pH and NO_3^-:NH_4^+ ratios (expressed as percent NO_3^-) is presented in Table 3 and Fig. 1. The fitted regression line, medium pH = 3.6957 + 0.0244*(%NO_3^-) explained 92% of the total variation in pH and was highly significant ($p < 1\%$). The close correlation between pH and the NO_3^-:NH_4^+ ratio is consistent with known mechanisms of inorganic N uptake in plants and the maintenance of electroneutrality. Inorganic N uptake can strongly influence pH since the uptake of NO_3^- and NH_4^+ changes the pH in opposite directions. The absorption of the NO_3^- anion results in an increase in pH to maintain charge neutrality, generally by the excretion of HCO_3^-. Conversely, absorption of NH_4^+ cations results in the production of H^+ which are excreted into the medium, thereby lowering the pH and further reducing cation uptake by competitive effects (Kirkby & Mengel 1967).

Regression analysis of % gain in fresh weight and pH is presented in Table 3. Fitting a curve revealed a highly significant fit for a quadratic model that accounted for 52% of the total variation, while the linear model accounted for only 26%. The similarity in the response of increase in fresh weight to %NO_3^- or medium pH (i.e. $r^2 = 0.54$ vs $r^2 = 0.52$; Table 3) suggests that the variation explained by %NO_3^- is probably due to the indirect effect of %NO_3^- on pH control.

Effect on somatic embryogenesis

The influence of the NO_3^-:NH_4^+ ratio on subsequent embryogenesis of the callus was tested by subculturing some callus from each treatment onto embryogenesis induction medium. The callus in all treatments differentiated into somatic embryos within 4–6 weeks, indicating that these treatments did not adversely affect the ability of the callus to undergo somatic embryogenesis. No further attempt was made to regenerate plants from the embryos.

Conclusion

The optimum NO_3^-:NH_4^+ ratio for growth of sweet orange callus is between 50% and 90% NO_3^-. Nitrate levels outside this range resulted in a significant reduction in callus growth. At NO_3^- concentrations of 90% or greater the callus is whiter than callus grown at lower NO_3^- levels. Medium pH in unbuffered media can be accurately controlled and predicted solely by varying the NO_3^-:NH_4^+ ratio.

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