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Some morphogenic effects of sodium sulfate on tobacco callus

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Abstract. Callus cultures of *Nicotiana tabacum* L cv. Wisconsin 38 were initiated and grown on shoot-forming (SF) and callus proliferation (CP) medium with or without Na_2SO_4. Two cultures were maintained on SF medium with 0, 0.75, 1 or 1.5% Na_2SO_4 for 2.5 and 3.5 years. In the older culture only callus grown on salt formed shoots throughout the maintenance period, while in the younger culture the control responded best and Na_2SO_4 was inhibitory. Callus from the older culture which had been grown on salt continued to form shoots in the absence of salt. Na_2SO_4 caused adventitious shoot formation in three cultures on CP medium. These shoots were present for 7 subcultures after removal of Na_2SO_4; but established, control callus, did not form shoots when transferred to Na_2SO_4. Callus initiated and maintained on NaCl or mannitol showed a slight increase in shoot initiation. On NaCl, Na_2SO_4 or mannitol, the tissue osmotic potential became more negative and proline concentration increased.

Introduction

Regeneration of shoots and roots from tobacco callus and explants is well established as a model system for studying organogenesis in vitro. Although growth regulators are critical factors controlling organ formation in this species [16], many non-hormonal factors are also regulatory [18, 20]. We have observed that sodium sulfate promotes shoot formation in tobacco callus. This salt was shown to induce adventitious shoot formation on a medium designed for callus proliferation [15], and enhance shoot formation on a medium supporting caulogenesis [14].

The present study was undertaken to determine the stability and reproducibility of these salt effects, whether they were correlated with water relations or metabolite concentrations, and if the responses were specific to Na_2SO_4.

Materials and methods

Initiation and maintenance of cultures

Stock callus cultures were initiated [19] from pith explants of *Nicotiana tabacum* cv Wisconsin 38 on either SF medium (shoot-forming medium; [14]) or CP medium (callus proliferation medium; [15]) supplemented with 0, 0.75, 1 or 1.5% (w/v; 0, 52.5, 70, 105 mM, respectively) Na_2SO_4. Cultures maintained without supplements are referred to as control cultures. Cultures were also established on CP medium supplemented with NaCl (0.5 or 1%; 85 or 170 mM) or mannitol (3 or 6%; 165 or 330 mM). Cultures established on SF medium were maintained in the light (16 h photoperiod; photon fluence rate ca. 80 μmol $m^{-2} s^{-1}$; 380–800 nm) and dark, while those on CP medium were kept in the light only. All cultures were maintained at 27 $\pm$ 1 °C.

All lines were subcultured at monthly intervals and 20 callus pieces were kept of each. Shoot formation was scored at each subculture. Shoot-forming pieces (described in Results) were easily distinguished on both SF and CP medium, and percentage shoot formation was calculated (number of callus pieces with shoots [18]). Average percentage shoot formation for any one line was calculated as the mean of all % shoot formation values recorded for that line; up to the last subculture adventitious shoots were observed. On both SF and CP medium callus was subcultured from all types of tissue and there was no deliberate selection of explants with shoots for subculture.

In various experiments callus from the different stocks were transferred to media of different composition (see Results). In these experiments there were 15–20 replicate callus pieces per treatment and callus was grown for one month. After this, shoot formation was assessed in the same way as described for stocks, and pieces were either subcultured to media of the same composition or used for determination of fresh weight, FW:DW (fresh weight:dry weight) or metabolite concentration.

Determination of water relations and metabolite concentration

Osmotic potentials were measured as previously described [3], with an equilibration time of 3 hours. In shoot-forming pieces, callus adjacent to, but not including, shoots was taken. For metabolite measurement fresh callus was frozen in liquid nitrogen and homogenized in 4 ml methanol: chloroform:water (12:5:1). After partition against chloroform and water, proline was determined from the upper layer as previously described [4]. Reducing sugars were assayed according to Somogyi [17], and sucrose calculated by comparison of aliquots incubated with or without invertase.

Table 1. The effect of Na_2SO_4 on shoot formation in tobacco callus maintained on a shoot-forming medium in the light and dark.

Culture	Level of Na_2SO_4 during maintenance (%)	Last subculture shoots were observed		Mean percentage shoot formation during shoot forming period	
		Light	Dark	Light	Dark
1. Established for	0	31	29	83.1	50.0
31 subcultures,	0.75	31	30	75.5	42.5
on all Na_2SO_4	1	30	28	63.9	39.6
levels.	1.50	27	18	36.5	32.8
2. Established for	0	17	41	25.6	46.2
41 subcultures,	0.75	39	39	59.2	59.0
on all Na_2SO_4	1	41	40	78.6	53.8
levels.	1.50	41	41	56.8	55.4

Results

Effects of Na_2SO_4 on callus maintained on SF medium

The two lines used in our initial study [14] have now been maintained for 31 and 41 monthly subcultures. Table 1 summarizes the effect of Na_2SO_4 on shoot formation in these two cultures. In the older culture, shoot formation was both retained and enhanced in light-grown (but not dark-grown) callus maintained on SF medium supplemented with Na_2SO_4. If long-term light-grown callus was transferred from no Na_2SO_4 to SF medium containing Na_2SO_4, no shoots were formed. However, Na_2SO_4-grown callus continued to form shoots when maintained in the absence of the salt. In the second

Table 2. The effect of Na_2SO_4 on shoot formation in tobacco callus maintained on callus proliferation medium in the light. Cultures were maintained for 44, 45 and 16 monthly subcultures (cultures 1–3 respectively).

	Levels of Na_2SO_4 during maintenance (%)	Culture 1	Culture 2	Culture 3
Last subculture	0	4	3	2
shoots were	0.75	36	20	11
observed	1	38	10	16
	1.50	no shoot formation	8	no culture established
Mean percentage	0	4.3	20.3	32.0
shoot formation	0.75	26.2	32.5	17.0
during shoot	1	28.7	27.0	22.6
forming period	1.50	–	15.0	–

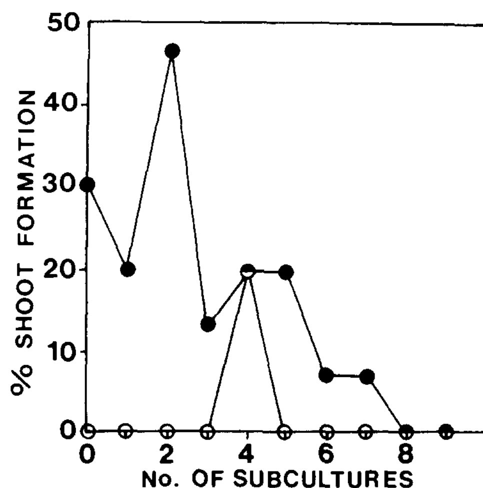

Fig. 1. Percentage shoot formation in eight subculture-old tobacco callus maintained on callus proliferation medium with 1% Na_2SO_4 and then transferred to no salt (●) for a further 9 subcultures and in eight subculture-old control callus transferred to 1% NA_2SO_4 (○) for 9 subcultures.

(younger) culture, shoot formation was retained in the absence of Na_2SO_4 throughout the maintenance period and salt inhibited shoot formation in both the light and dark (Table 1). Shoot-forming callus was healthy, bright-green and had numerous shoot primordia, but few well-formed shoots.

Morphogenic effects of Na_2SO_4 in callus maintained on CP medium

In callus maintained on CP medium the effect of Na_2SO_4 was to promote adventitious shoot formation (Table 2). On average 20–30% of explants formed shoots. Percentage shoot formation was 50–100% during the first 8 to 10 subcultures on salt-containing medium and gradually declined during maintenance. Most explants (60–70%) produced single shoots, with obvious leaves. The callus which formed shoots was dark-green and always had friable edges. Adventitious roots were observed in approximately 5–10% of all explants, regardless of whether adventitious shoots were also formed. Salt-grown callus maintained in the absence of Na_2SO_4 continued to form shoots, while only once was shoot formation observed in control callus during exposure to Na_2SO_4 for 9 subcultures (Fig. 1).

Morphogenic effects of NaCl and mannitol in callus maintained on CP medium

Shoot formation was enhanced in callus initiated and maintained on either NaCl or mannitol (Table 3). Callus grown in 1% NaCl for 6 or 9 subcultures

Table 3. Percentage shoot formation in tobacco callus maintained on callus proliferation medium supplemented with no salt, NaCl or mannitol.

Number of subcultures	No salt[a]	NaCl (%)		Mannitol (%)	
		0.5	1	3	6
1	15	55	44	7	0
2	10	60	25	45	27
3	23	25	0	20	0
4	6	30	30	0	0
5	0	0	40	0	10
6	0	0	0	0	21
7	0	5	0	0	29
8	0	0	5	0	16
9	0	0	0	0	0
10	0	0	0	10	0
11–16	0	0	0	–[b]	–

[a] Mean of combined results for 4 experiments.
[b] No culture.

did not form shoots if transferred to 1% Na_2SO_4 for one passage (data not shown). Ten month-old callus previously grown in the absence of salt did not form shoots if maintained on CP medium supplemented with 2, 4, 6 or 8% mannitol for two subcultures, whereas shoot formation persisted on all levels of mannitol except 8%, if the callus was previously maintained on 1% Na_2SO_4 (data not shown).

Growth, water relations and solute concentrations in callus grown on CP medium with salt or mannitol

Callus maintained on Na_2SO_4 grew better on this salt than callus from control cultures (Fig. 2). Fresh weights in the absence of salt were similar, and 2% Na_2SO_4 was completely toxic, in both cases. Callus maintained on Na_2SO_4 had a higher FW:DW ratio than control callus on all Na_2SO_4 levels and on CP medium containing NaCl, KCl or K_2SO_4 (Fig. 2).

Osmotic potentials in callus continuously maintained on 1% NaCl or 1% Na_2SO_4 were approximately -16 and -13 to -14 bars respectively. The difference between shoot-forming callus and non-morphogenic callus on 0, 0.5 and 1% Na_2SO_4 was 3.4, 1.2 and 1.0 bars respectively. Fresh weight:dry weight decreased and osmotic potential became more negative in callus grown on mannitol, but these changes were smaller if callus was previously maintained on Na_2SO_4 (Fig. 3). The osmotic potential of callus from shoot-forming explants on mannitol-containing medium was more positive than callus from explants without adventitious shoots (Fig. 3).

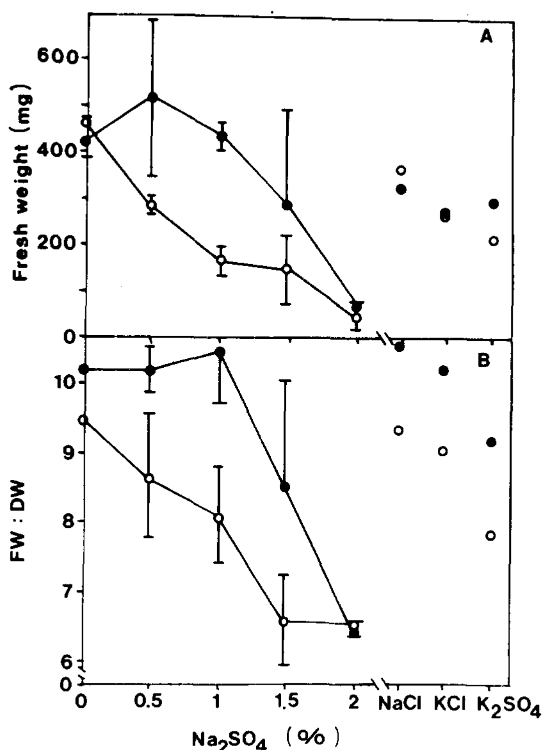

Fig. 2. Fresh weights (A) and FW:DW (B) of control tobacco callus (○) and callus maintained in 1% Na_2SO_4 (●) (both 9 subculture-old stocks) after transfer to callus proliferation medium containing 0–2% Na_2SO_4 or NaCl, KCl or K_2SO_4 equimolar to 1% Na_2SO_4. Fresh weights are the mean of 15 replicates and for Na_2SO_4 treatments the means of two separate experiments (± S.E.) are shown. Initial inoculum weights were 30–50 mg fresh weight.

Table 4 shows that with one exception (8 month-old Na_2SO_4 maintained callus), control and Na_2SO_4-maintained callus contained less reducing sugars and sucrose on Na_2SO_4 than on no salt. In both control and salt-grown callus proline concentration was elevated in callus grown on Na_2SO_4 (Table 4). Proline concentration also increased in control or Na_2SO_4-grown callus transferred to mannitol. Maximum increases were on 4% mannitol, and were 225% and 185% for control and Na_2SO_4-grown sources respectively, on a dry weight basis. There were no significant differences in proline, reducing sugars or sucrose concentration when callus from explants with and without adventitious shoots was compared.

Discussion

When tobacco cells are grown on salt they lose water [2], experience an increase in water and osmotic potentials [2, 6, 15], and accumulate ions and

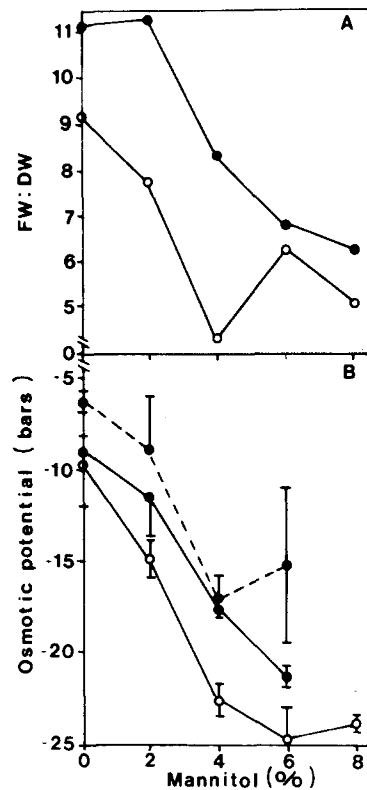

Fig. 3. FW:DW (A) and osmotic potential (B) of control (○) and Na_2SO_4-grown (●) tobacco callus transferred to callus proliferation medium containing 0–8% mannitol. Both stocks were ten subcultures old at the start of the experiment. Tissue from the salt-maintained source was sampled from both non shoot-forming (●——●) and shoot-forming (●- - -●) callus pieces. Each osmotic potential value is the mean ± S.E. of duplicate determinations.

metabolites such as proline and reducing sugars [6, 21]. Proliferation of salt tolerant cells, which may also have other phenotypic characteristics, is also favored. A key aim of this study was to determine if any of these events could be correlated to adventitious shoot formation in Na_2SO_4-grown tobacco callus. Studies with NaCl-grown callus [2, 6, 21, 22] have not revealed a similar organogenic response. Our physiological studies were undertaken using CP medium, since addition of Na_2SO_4 promotes adventitious shoot initiation [15].

An increase in osmotic potential and in proline concentration, both of which occur on sodium sulfate, are known to be events associated with organogenesis in tobacco callus [3, 13, 18]. However, this also occurred in established control callus, incapable of shoot formation, when it was exposed to Na_2SO_4. In addition, mannitol also caused these changes, and

Table 4. Proline, reducing sugars and sucrose concentration in tobacco callus grown on callus proliferation medium containing 0 or 1% Na_2SO_4 for 1 month. Callus had previously been maintained on no salt (for 5, 6 or 8 subcultures), or 1% Na_2SO_4 (for 6 or 8 subcultures).

Culture		Proline (mg.g DW^{-1})		Reducing Sugars (mg glucose equivs. g DW^{-1})		Sucrose (mg.g DW^{-1})	
Supplement	No. of Subcultures	No Na_2SO_4	1% Na_2SO_4	No Na_2SO_4	1% Na_2SO_4	No Na_2SO_4	1% Na_2SO_4
No salt	5	22.2 ± 10.5[a]	56.0 ± 5.1	9 ± 1	6 ± 1	138 ± 5	84 ± 2
	6	24.9 ± 4.7	40.3 ± 3.8	30 ± 8	8 ± 2	211 ± 23	134 ± 13
	8	15.1 ± 2.3	50.0 ± 6.9	5 ± 1	5 ± 2	288 ± 26	215 ± 21
	(mean)	(20.7)	(48.8)	(15)	(6)	(212)	(144)
1% Na_2SO_4	6	6.1 ± 1.7	31.2 ± 3.5	98 ± 5	12 ± 3	277 ± 7	133 ± 15
	8	27.8 ± 4.0	47.5 ± 9.2	49 ± 32	7 ± 3	89 ± 6	160 ± 16
	(mean)	(17.0)	(39.4)	(74)	(10)	(183)	(147)

[a] Mean ± S.E. of 3 replicates.

shoot-forming and non shoot-forming callus from Na_2SO_4-grown stocks behaved similarly with respect to these parameters during caulogenesis. The quite specific Na_2SO_4 effect does not appear therefore, to be due to unique changes in osmotic potential or proline concentration, although these may provide conditions favorable for shoot formation. This is indicated by the beneficial effect of NaCl and mannitol. The specificity of Na_2SO_4 may be explained if it acts independently of any physiological effects, but induces shoot-forming capacity or is a selective agent favoring proliferation of cells with such capacity. In the latter case, such cells may exist early in culture (shoot formation is indeed observed over the first few subcultures in the absence of Na_2SO_4), but be lost in the absence of the selection pressure.

The effect of Na_2SO_4 was not permanent and there was a gradual decline in shoot initiation. It is likely that genomic changes accompanying long-term subculture could lead to a permanent loss of shoot-forming capacity. The possibility of metabolites unique to callus grown on Na_2SO_4 must still be addressed, as should the possibility that Na_2SO_4 could be inducing an effect similar to cytokinin autotrophy. The incidence of single adventitious shoots, reversion to non shoot formation in the absence of stress, and variation in the rate of initiation are all characteristics of such an induction of cytokinin autotrophy [9–12]. If sodium sulfate does act in this way, the results also indicate that induction was only possible in young callus, as shoots could not be induced in established control callus. Finally, our observations do not appear to be confined to tobacco, as indicated by a number of recent reports where water and salt stress have been shown to promote organogenesis in vitro [1, 5, 7, 8]. However, the mechanism for this phenomenon remains to be determined.

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