

## Optimization of Medium for Growing the Aquatic Carnivorous Plant *Aldrovanda vesiculosa* In Vitro

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### Abstract

In Polish plants of *Aldrovanda vesiculosa* growing in a half-strength B5 medium with different KNO<sub>3</sub> concentrations, the medium with 1000 mg l<sup>-1</sup> KNO<sub>3</sub> was the best in all characteristics. The addition of 100, 300 and 1000 mg l<sup>-1</sup> peptone to half-strength B5 medium with 500 mg l<sup>-1</sup> KNO<sub>3</sub> for Polish plants led to a step-wise increase of all growth characteristics but usually only the effect of 300 and 1000 mg l<sup>-1</sup> peptone was statistically significant. No clear relationship was found between growth characteristics and KNO<sub>3</sub> concentration in a half-strength B5 for Japanese plants. The addition of 100, 300 and 1000 mg l<sup>-1</sup> peptone to a half-strength B5 medium with 500 mg l<sup>-1</sup> KNO<sub>3</sub> led to a two- to threefold increase in the number of shoot apices. pH values below 3.0 in used B5 media were not toxic for the growth of *Aldrovanda in vitro*. In both strains of *Aldrovanda* tested, the full-strength B5 medium with 2500 mg l<sup>-1</sup> KNO<sub>3</sub> appeared to be excessively concentrated for optimal growth.

**Key words:** *Aldrovanda vesiculosa*, aseptic culture, carnivorous plant, Polish strain.

### Abbreviations

SE, standard error.

*Aldrovanda vesiculosa* L. (Droseraceae) is a critically endangered and rare aquatic carnivorous plant. It is rootless, free-floating, and grows just below the surface in shallow standing dystrophic waters (Lloyd, 1942; Adamec, 1995). Kondo *et al.* (1997) developed a method of growing of *Aldrovanda* strains from Japan and Eastern Poland in a sterile *in vitro* culture. They used Gamborg's B5 liquid medium (B5; Gamborg *et al.*, 1968) with 2% sucrose and 2500 mg l<sup>-1</sup> KNO<sub>3</sub>. Since this medium is rather concentrated, Adamec and Pásek (2000) tried to modify it and preliminarily found a half-strength B5 medium with only 500 mg l<sup>-1</sup> KNO<sub>3</sub> to be the best for *Aldrovanda* growth and branching of shoots. However, pH values in all exhausted B5 media were very low, from 2.94 to 3.41. These pH values are far the optimum for the growth of *Aldrovanda*, which was found to be about 4.5 in a rather diluted mineral nutrient solution (Kamiński, 1987) or about 6.5 at natural sites (Adamec, 1997a). The acidification of B5 media could be caused by

the fact that *Aldrovanda* greatly prefers the uptake of NH<sub>4</sub><sup>+</sup> to NO<sub>3</sub><sup>-</sup> (Adamec, 2000). Moreover, aquatic carnivorous plants can take up a great deal of their total N gain in the form of organic substances (for a review see Adamec, 1997b).

In this paper, we have investigated the effect of KNO<sub>3</sub> and N-containing organic substances in B5 medium on the growth of *A. vesiculosa* in an aseptic culture *in vitro*.

*Aldrovanda* plants from Eastern Poland (Lake Długie) and Japan (Hozoji Pond, Hanyu City; Kondo *et al.*, 1997) used for the experiments were pre-cultured in one-fourth strength liquid medium with 2% sucrose on a rotary cultivation apparatus (2 cycles per min) at 26 ± 0.5 °C under 505 lux continuous fluorescent illumination. The following modifications of the standard B5 medium (in mg l<sup>-1</sup>: KNO<sub>3</sub>, 2500; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 134.0; NaH<sub>2</sub>PO<sub>4</sub>, 130.5; CaCl<sub>2</sub>·6H<sub>2</sub>O, 223.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 250.0; FeSO<sub>4</sub>·7H<sub>2</sub>O, 27.8; Na<sub>2</sub>EDTA, 37.3; H<sub>3</sub>BO<sub>3</sub>, 3.0; MnSO<sub>4</sub>, 10.0; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 2.0; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.025; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.025; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.25; KJ, 0.75; inositol, 100; thiamine, 10.0; nicotinic acid, 1.0; pyridoxine, 1.0) were tested. A, full-strength standard B5 + 2500 mg l<sup>-1</sup> KNO<sub>3</sub>; B, 50% B5 + 200 mg l<sup>-1</sup> KNO<sub>3</sub>; C,

**Table 1** The *in vitro* growth of *Aldrovanda vesiculosa* from Eastern Poland in modified Gamborg B5 media after 22 days

Var.	Total shoot apices per tube after		Shoot length (cm)	Leaf whorls of main shoot	Dry weight per tube (mg)	Max. trap size (mm)	Final pH of medium (range)
	14 days	22 days					
A	6.3 ± 0.3 <sup>ab</sup>	6.3 ± 0.3 <sup>a</sup>	6.4 ± 0.4 <sup>a</sup>	26.9 ± 1.9 <sup>ab</sup>	65.3 ± 3.8 <sup>ac</sup>	3.0 ± 0.0 <sup>b</sup>	3.42(3.35–3.47) <sup>a</sup>
B	5.0 ± 0.0 <sup>a</sup>	8.00 ± 0.6 <sup>ac</sup>	6.4 ± 0.4 <sup>a</sup>	25.5 ± 3.0 <sup>a</sup>	72.0 ± 3.0 <sup>ac</sup>	3.0 ± 0.0 <sup>b</sup>	2.91(2.85–2.99) <sup>bd</sup>
C	4.7 ± 0.7 <sup>a</sup>	7.0 ± 0.6 <sup>ac</sup>	7.0 ± 0.2 <sup>a</sup>	27.3 ± 0.8 <sup>ab</sup>	64.7 ± 9.7 <sup>ac</sup>	3.0 ± 0.0 <sup>b</sup>	2.96(2.90–3.02) <sup>bd</sup>
D	5.0 ± 0.6 <sup>a</sup>	8.3 ± 0.3 <sup>ac</sup>	8.5 ± 0.1 <sup>bcd</sup>	33.4 ± 1.7 <sup>b</sup>	82.3 ± 7.6 <sup>ae</sup>	3.2 ± 0.2 <sup>bc</sup>	3.06(3.02–3.11) <sup>b</sup>
E	5.7 ± 0.3 <sup>ab</sup>	7.0 ± 0.0 <sup>ac</sup>	7.4 ± 0.2 <sup>ac</sup>	29.9 ± 0.6 <sup>ab</sup>	66.3 ± 5.0 <sup>ac</sup>	3.0 ± 0.0 <sup>b</sup>	2.85(2.80–2.88) <sup>cd</sup>
F	6.0 ± 0.6 <sup>ab</sup>	10.3 ± 0.9 <sup>bcd</sup>	9.0 ± 0.1 <sup>d</sup>	31.9 ± 0.7 <sup>ab</sup>	88.0 ± 7.9 <sup>adf</sup>	3.0 ± 0.0 <sup>b</sup>	2.93(2.90–2.96) <sup>bc</sup>
G	5.3 ± 0.3 <sup>a</sup>	12.7 ± 0.9 <sup>b</sup>	9.6 ± 0.1 <sup>de</sup>	33.8 ± 0.6 <sup>b</sup>	91.3 ± 3.7 <sup>ad</sup>	3.3 ± 0.2 <sup>bc</sup>	2.93(2.89–2.96) <sup>bc</sup>
H	8.3 ± 0.7 <sup>b</sup>	14.0 ± 1.2 <sup>b</sup>	10.6 ± 0.3 <sup>c</sup>	35.2 ± 1.0 <sup>b</sup>	120.0 ± 7.5 <sup>bd</sup>	3.5 ± 0.0 <sup>c</sup>	3.06(2.99–3.14) <sup>b</sup>
I	4.3 ± 0.3 <sup>a</sup>	6.7 ± 0.3 <sup>ad</sup>	6.4 ± 0.1 <sup>a</sup>	28.5 ± 1.4 <sup>ab</sup>	53.3 ± 2.4 <sup>cef</sup>	3.0 ± 0.0 <sup>b</sup>	3.05(3.04–3.06) <sup>b</sup>
J	5.0 ± 0.6 <sup>a</sup>	5.0 ± 0.6 <sup>a</sup>	5.9 ± 0.2 <sup>a</sup>	24.5 ± 0.4 <sup>a</sup>	37.7 ± 2.0 <sup>c</sup>	2.5 ± 0.0 <sup>a</sup>	3.47(3.46–3.48) <sup>a</sup>

A, full-strength B5 + 2500 mg l<sup>-1</sup> KNO<sub>3</sub>; B, 50% B5 + 200 mg l<sup>-1</sup> KNO<sub>3</sub>; C, 50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub>; D, 50% B5 + 1000 mg l<sup>-1</sup> KNO<sub>3</sub>; E, 50% B5 + 0 mg l<sup>-1</sup> KNO<sub>3</sub>; F, 50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub> + 100 mg l<sup>-1</sup> peptone; G, 50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub> + 300 mg l<sup>-1</sup> peptone; H, 50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub> + 1000 mg l<sup>-1</sup> peptone; I, 50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub> + 20 mg l<sup>-1</sup> glycine; J, 10% B5 + 20 mg l<sup>-1</sup> KNO<sub>3</sub>. Mean values ± 1.S.E interval are stated in all cases. The data are means of three parallel test tubes. Within each column, the variants labelled by the same letters are not statistically significantly different at p<0.01.

50% B5 + 500 mg l<sup>-1</sup> KNO<sub>3</sub>; D, 50% B5 + 1000 mg l<sup>-1</sup> KNO<sub>3</sub>; E, 50% B5 + 0 mg l<sup>-1</sup> KNO<sub>3</sub>; F, 50% B5+500 mg l<sup>-1</sup> KNO<sub>3</sub>+100 mg l<sup>-1</sup> peptone; G, 50% B5+500 mg l<sup>-1</sup> KNO<sub>3</sub>+300 mg l<sup>-1</sup> peptone; H, 50% B5+500 mg l<sup>-1</sup> KNO<sub>3</sub>+1000 mg l<sup>-1</sup> peptone; I, 50% B5+500 mg l<sup>-1</sup> KNO<sub>3</sub>+20 mg l<sup>-1</sup> glycine; J, 10% B5+20 mg l<sup>-1</sup> KNO<sub>3</sub>. All media contained 2% sucrose and their pH was adjusted to 5.5 (NaOH or HCl) before autoclaving.

Four non-branched 2.0 ± 0.2 cm long apical segments from shoots of Eastern Polish (mean plant dry weight 5.1 mg) or 1.5 ± 0.2 cm long segments of Japanese *Aldrovanda* plants (mean plant dry weight 0.81 mg) were inserted aseptically into each of 3 cm diameter, 100 ml test tubes into 40 ml of the media. Each variant of the media consisted of three parallel test tubes for each *Aldrovanda* strain. The experimental plants were grown under the same conditions as above. Shoot apices of the Polish plants were counted without opening the test tubes after 14 days of growth. The growth experiment was terminated after 22 days for Polish plants and 28 days for Japanese plants. The following characteristics were estimated: total shoot apices per tube, shoot length, number of adult leaf whorls on main shoots, maximum trap size, dry weight per tube, and pH of used media. Browned senescent leaf whorls were discarded. The data were expressed as mean (± 1.S.E) of three parallel test tubes and analyzed using ANOVA (Tukey HSD test).

During the whole growth experiment, plant dry weight increased on average by 1.9–5.9 fold in Polish plants (**Table 1**) and by 2.2–5.2 fold in Japanese ones (**Table 2**). In Polish plants growing both in the full-strength standard B5 and in one-tenth-strength B5 medium with only 20 mg l<sup>-1</sup> KNO<sub>3</sub>, branching of shoots ceased as early as 14 days of growth, while all the other media were able to further support statistically significantly (at P<0.05) branching of shoots. In Polish plants growing in half-strength B5 medium with different KNO<sub>3</sub> concentrations, the medium with 1000 mg l<sup>-1</sup> KNO<sub>3</sub> was the best in all characteristics though the statistical significance was rather weak (**Table 1B–E**).

The addition of 100, 300 and 1000 mg l<sup>-1</sup> peptone to half-strength B5 medium with 500 mg l<sup>-1</sup> KNO<sub>3</sub> for Polish plants led to a step-wise increase of all growth characteristics but usually only the effect of 300 and 1000 mg l<sup>-1</sup> peptone was statistically significant (**Table 1C, F–H**). The variant with 1000 mg l<sup>-1</sup> peptone was clearly the best of all tested media for the growth of Polish plants. The addition of 20 mg l<sup>-1</sup> of glycine was without any effect on plant growth (**Table 1C, I**). Similarly, addition of certain amino acids had no effect or a negative effect on the growth of *Drosera rotundifolia in vitro* (Simola, 1978). In Polish plants except for the full- and one-tenth-strength B5 medium, mean pH values in all other used media were between 2.85

**Table 2** The *in vitro* growth of *Aldrovanda vesiculosa* from Japan in modified Gamborg B5 media after 28 days

Var.	Total shoot apices per tube	Shoot length (cm)	Leaf whorls of main shoot	Dry weight per tube (mg)	Max. trap size (mm)	Final pH of medium (range)
A	7.7 ± 3.7 <sup>a</sup>	2.0 ± 0.04 <sup>a</sup>	2.3 ± 0.2 <sup>a</sup>	7.7 ± 1.7 <sup>a</sup>	2.3 ± 0.1 <sup>a</sup>	3.76(3.64–3.96) <sup>ac</sup>
B	6.7 ± 0.3 <sup>c</sup>	2.6 ± 0.1 <sup>bc</sup>	4.9 ± 0.2 <sup>ab</sup>	11.7 ± 1.9 <sup>ab</sup>	2.1 ± 0.1 <sup>ab</sup>	3.36(3.28–3.35) <sup>ag</sup>
C	15.3 ± 4.2 <sup>abf</sup>	2.6 ± 0.1 <sup>bc</sup>	5.1 ± 0.1 <sup>ab</sup>	12.7 ± 0.5 <sup>ab</sup>	1.9 ± 0.1 <sup>ab</sup>	3.34(3.29–3.38) <sup>ag</sup>
D	11.3 ± 3.5 <sup>ab</sup>	2.5 ± 0.03 <sup>b</sup>	4.6 ± 0.2 <sup>ab</sup>	10.3 ± 1.5 <sup>ab</sup>	2.0 ± 0.1 <sup>ab</sup>	3.43(3.36–3.53) <sup>a</sup>
E	18.7 ± 5.0 <sup>abc</sup>	3.0 ± 0.1 <sup>bc</sup>	6.1 ± 1.0 <sup>ab</sup>	12.0 ± 0.6 <sup>ab</sup>	1.7 ± 0.1 <sup>ab</sup>	3.14(3.06–3.26) <sup>bg</sup>
F	29.7 ± 5.2 <sup>bdf</sup>	2.7 ± 0.1 <sup>bc</sup>	6.4 ± 1.0 <sup>ab</sup>	13.9 ± 2.9 <sup>ab</sup>	2.0 ± 0.1 <sup>ab</sup>	3.49(3.34–3.62) <sup>ade</sup>
G	42.3 ± 2.9 <sup>dl</sup>	3.0 ± 0.1 <sup>c</sup>	8.4 ± 0.8 <sup>b</sup>	18.3 ± 0.3 <sup>b</sup>	1.9 ± 0.2 <sup>ab</sup>	3.60(3.53–3.67) <sup>adf</sup>
H	32.7 ± 0.9 <sup>cdef</sup>	2.8 ± 0.1 <sup>bc</sup>	6.2 ± 1.3 <sup>ab</sup>	11.7 ± 1.2 <sup>ab</sup>	2.0 ± 0.1 <sup>ab</sup>	4.47(4.33–4.59) <sup>ceh</sup>
I	14.0 ± 1.7 <sup>abc</sup>	2.7 ± 0.1 <sup>bc</sup>	4.3 ± 0.4 <sup>ab</sup>	12.0 ± 0.2 <sup>ab</sup>	1.5 ± 0.1 <sup>b</sup>	3.56(3.46–3.72) <sup>adh</sup>
J	17.3 ± 1.3 <sup>abc</sup>	2.7 ± 0.1 <sup>bc</sup>	6.8 ± 1.0 <sup>ab</sup>	17.0 ± 0.6 <sup>b</sup>	1.5 ± 0.3 <sup>ab</sup>	3.38(3.37–3.39) <sup>ad</sup>

For the description of the media and further details see **Table 1**.

and 3.06 (**Table 1**). Higher KNO<sub>3</sub> concentrations corresponded to slightly higher pH values.

Growth characteristics of Japanese plants in various media were slightly different from those of Polish plants (**Table 2**). The worst variant was clearly the standard full-strength B5 medium. No clear relationship was found between growth characteristics and KNO<sub>3</sub> concentration in a half-strength B5 (**Table 2B–E**). However, within these variants, zero KNO<sub>3</sub> concentration was the best. Similar growth was attained in one-tenth-strength B5. The addition of 100, 300 and 1000 mg l<sup>-1</sup> peptone to a half-strength B5 medium with 500 mg l<sup>-1</sup> KNO<sub>3</sub> led to a two- to threefold increase in the number of shoot apices but these differences were usually not statistically significant (**Table 2C, F–H**). Out of all variants, that with 300 mg l<sup>-1</sup> peptone was clearly the best (number of shoot apices and leaf whorls, shoot length, and dry weight) for the growth of Japanese plants. The addition of 20 mg l<sup>-1</sup> of glycine was without any effect on plant growth (**Table 2C, I**). pH values in used media ranged from 3.14 to 3.76, except for the medium with 1000 mg l<sup>-1</sup> peptone (pH 4.47).

Generally, Japanese *Aldrovanda* plants in a rotary *in vitro* culture are much smaller and thinner than E Polish plants (Kondo *et al.*, 1997), and the same relation was confirmed by the present results (cf. **Table 1, 2**). According to these authors, this difference is genetically based. However, in a stagnant *in vitro* culture in a half-strength B5 medium, their size and features are the same (Pásek and Adamec, unpublished).

The optimal KNO<sub>3</sub> concentration in half-strength B5 medium for growth of both Polish and Japanese *Aldrovanda* plants may be 500–1000 mg l<sup>-1</sup>. Such high concentrations are not necessary for total

mineral N uptake by the plants but they partly counterbalance medium acidification due to dominant NH<sub>4</sub><sup>+</sup> uptake. However, KNO<sub>3</sub> concentration could also have a direct effect on organogenesis as recently reported by Idei and Kondo (1998) in carnivorous *Utricularia praelonga* grown in a shoot primordium culture. The preference of Japanese *Aldrovanda* plants for one-tenth-strength diluted B5 medium or for low KNO<sub>3</sub> concentration (**Table 2**) may not represent specific properties of this strain but it may reflect the fact that the media were not exhausted at the end of the growth experiment, as opposed to Polish plants. During the growth experiment, small Japanese plants increased their dry biomass by only 4–15 mg per tube, while robust Polish plants did so by 18–100, on average ca. 50 mg per tube. In 40 ml of KNO<sub>3</sub>-free half-strength B5 medium in one tube, there was only 0.57 mg of NH<sub>4</sub><sup>+</sup>-N available. The mean 50-mg growth increment could contain ca. 0.60 mg N and 1.1 mg K in the biomass (ca. 1.2 % N and 2.2 % K in dry biomass; Adamec and Pásek, 2000). It means that the growth of Polish plants in KNO<sub>3</sub>-free a half-strength B5 or a tenth-strength B5 medium + 20 mg l<sup>-1</sup> KNO<sub>3</sub> (**Table 1E, J**) was evidently limited by a shortage of N, but much more so by low levels of K<sup>+</sup> in the tubes. Thus, the advantage of intermediate KNO<sub>3</sub> concentrations (500–1000 mg l<sup>-1</sup>) in half-strength B5 medium was based on much greater N and K<sup>+</sup> supplies supporting both greater growth increments and reaching higher pH values. However, pH values below 3.0 were not toxic for the growth of *Aldrovanda in vitro*. In both strains of *Aldrovanda* tested, the full-strength B5 medium with 2500 mg l<sup>-1</sup> KNO<sub>3</sub> appeared to be excessively concentrated for optimal growth (**Table 1, 2**; Adamec and Pásek, 2000).

An addition of peptone has shown to be positive for the growth of the both *Aldrovanda* strains. The peptone concentrations of 300, 300 and 1000 mg l<sup>-1</sup> were the best in the both strains (**Table 1, 2**). Since peptone as an extract of beef contains ca. 16% of organic N the relatively large growth enhancement due to the addition of peptone proves the great capacity of *Aldrovanda* for the uptake of nitrogenous organic substances instead of mineral forms of N (cf. Adamec, 1997b).

The present result can promise rapid micropropagation of *Aldrovanda vesiculosa* of the world.

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