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Acetylcholine causes rooting in leaf explants of in vitro raised tomato (*Lycopersicon esculentum* Miller) seedlings

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Abstract

The animal neurotransmitter acetylcholine (ACh) induces rooting and promotes secondary root formation in leaf explants of tomato (*Lycopersicon esculentum* Miller var. Pusa Ruby), cultured in vitro on Murashige and Skoog's medium. The roots originate from the midrib of leaf explants and resemble taproot. ACh at 10^{-5} M was found to be the optimum over a wide range of effective concentrations between 10^{-7} and 10^{-3} M. The breakdown products, choline and acetate were ineffective even at 10^{-3} M concentration. ACh appears to have a natural role in tomato rhizogenesis because exogenous application of neostigmine, an inhibitor of ACh hydrolysis, could mimic the effect of ACh. Neostigmine, if applied in combination with ACh, potentiated the ACh effect.

Keywords: Acetylcholine; Acetylcholinesterase; *Lycopersicon esculentum*; Neostigmine; Non-neuronal; Rhizogenesis; Tomato

Introduction

Acetylcholine (ACh) and its metabolizing enzymes are ubiquitously present in animals as well as in plants (Horiuchi et al. 2003). The role of ACh as a neurotransmitter in animals is well-established (Hoffman and Taylor, 2001). However, in recent years increasing evidence has accumulated to suggest non-neuronal functions for ACh, e.g. in regulation of morphogenetic cell movements, cell proliferation, growth and differentiation (Lauder and Schambra, 1999; Soreq and Seidman, 2001; Wessler et al., 2001; Cousin et al., 2005). In plants, ACh affects changes in electric potentials as well as several physiological functions (see for reviews Tretyn and Kendrick, 1991; Roshchina, 2001). However, no investigation has yet been reported concerning the effect of acetylcholine on morphogenesis in explants cultured in vitro. In the present study, we investigated the effect of acetylcholine and its breakdown products, choline and acetate, as well as neostigmine (Nst), an inhibitor of ACh breakdown, on morphogenic behaviour of leaf explants of tomato cultured on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962). Tomato was chosen as an experimental system because (a) its morphogenic behaviour in vitro is well known (Sink and Reynolds, 1986), and (b) the tomato family, Solanaceae contains ACh, the ACh hydrolysing enzyme acetylcholinesterase (AChE) as well as several alkaloids that are known to affect ACh system in animals (Roshchina, 2001).

Materials and methods

Plant material

Seeds of tomato, *Lycopersicon esculentum* Miller var. Pusa Ruby (National Seeds Corporation, New Delhi) were soaked in distilled water for an hour, surface sterilized with 1% (v/v) Polysan (Polypharma, Mumbai), and 0.1% (w/v) $HgCl_2$ (E. Merck, Mumbai), followed by rinsing with rectified spirit and sterilized distilled water. Seeds were germinated on Knop's medium containing 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. at 1.02 kg/cm^2. For the in vitro culture of plants, leaves from 30-day-old in vitro raised seedlings were used in all the experiments. Only the top two (distal) leaves were cultured.

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Leaves (approximately 0.5 cm) were trimmed at margins and cultured on MS basal medium alone as well as supplemented individually with 10^{-9} to 10^{-3} M ACh, choline, acetate or Nst. ACh and Nst were filter-sterilized. The media contained 3% sucrose and 0.8% agar. All the cultures were maintained at 28± 2 °C under white fluorescent light of 450–460 μW cm^{-2} programmed for 16 h photoperiod for 4 weeks. Explants in culture were subjected to chemical treatment from the time of inoculation. For each treatment, 48 explants were cultured and experiments were repeated thrice. The data of observation after 30 days of culture/continuous chemical treatment are reported here. Each datum represents an average of three experiments with 144 explants per treatment. The data were analyzed statistically and expressed as the mean±SE (within 95% confidence limit).

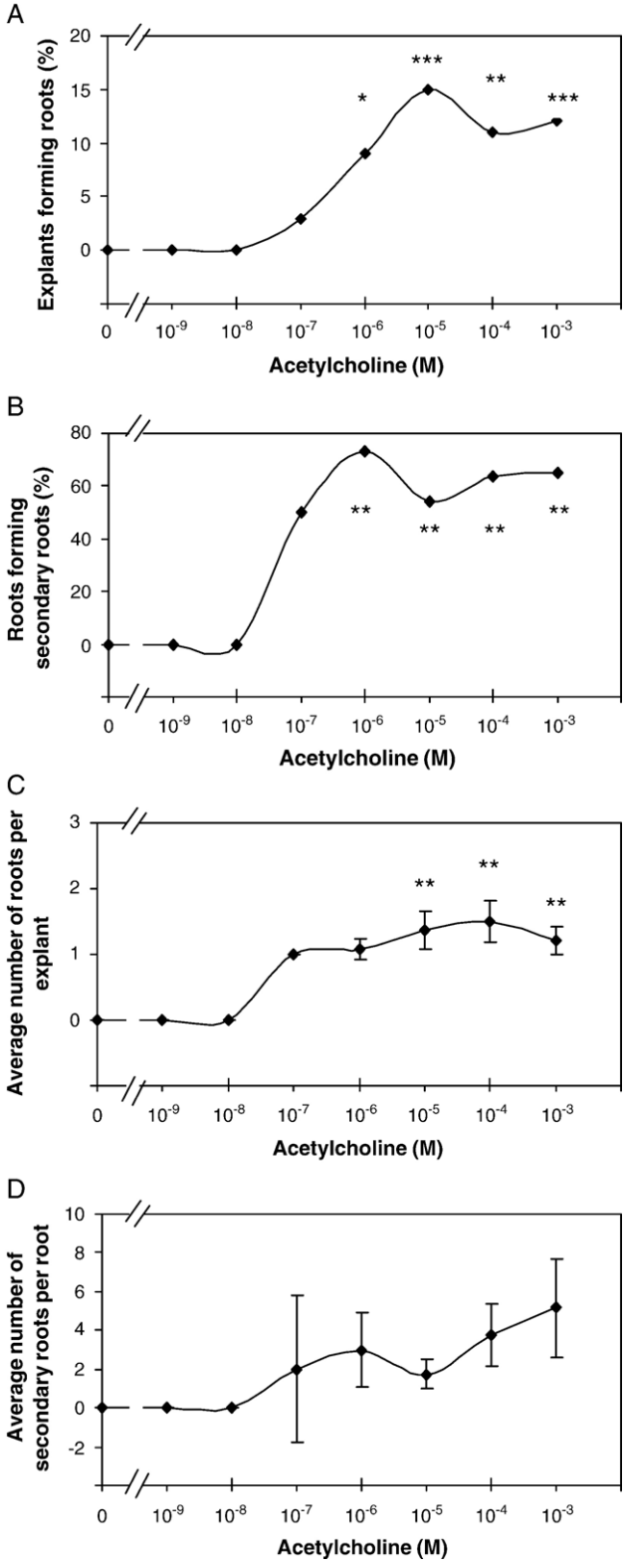

Fig. 1. A–D. Effect of ACh on morphogenic responses of leaf explants of tomato, *Lycopersicon esculentum* var. Pusa Ruby. ACh (10^{-9} to 10^{-3} M) was provided continuously in the culture medium from the first day of culture for 30 days. Leaf explants used here were excised from 30-day-old seedlings raised in vitro on MS basal medium. The data presented here are an average of three experiments with 144 explants per treatment. Error bars show±S.E.M. *Denotes significant differences between treatment and control ($P \leq 0.05$); **highly significant ($P \leq 0.01$); ***very highly significant ($P \leq 0.001$). A. Percentage of leaf explants forming roots. B. Percentage of roots forming secondary roots. C. Average number of roots formed per responding explant. D. Average number of secondary roots developed per root.

Estimation of activity of acetylcholinesterase (AChE)

Tomato cultures (4 g) were crushed in liquid nitrogen and homogenized in 8 ml of 4% (w/v) ammonium sulphate in 0.1 M K-Pi buffer (pH 7; 1:2 w/v); the homogenate was stirred for 20 min, filtered through cheesecloth; centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant was concentrated to dried powder form by lyophilisation and the proteins re-suspended in a small volume (2 ml) of 0.1 M K-Pi buffer, pH 7 and desalted by employing gel filtration on Sephadex G-25. Protein rich fractions were pooled, and tested for AChE activity (Ellman et al., 1961).

Statistical analysis of results

Data was subjected to univariate analysis of variance. Significant differences were established using post-hoc Tukey's HSD test.

Results

ACh (10^{-7} to 10^{-3} M) and neostigmine (10^{-7} to 10^{-3} M) induced rooting in excised leaves of *L. esculentum* var. Pusa Ruby cultured in vitro and promoted the number of roots per explant as well as formation of secondary roots (Figs. 1 and 2). A one-way ANOVA of data revealed that the there was significant induction of rooting as well as formation of secondary roots in explants treated with ACh or neostigmine (Fig 1A, B; Fig 2A, B). ACh was ineffective at 10^{-8} and 10^{-9} M. The optimum levels of ACh were 10^{-5} M for root induction (Fig. 1A), 10^{-4} M for increasing the number of roots (Fig. 1C), and 10^{-6} M for induction of secondary roots (Fig. 1B, D). Roots were always formed at the base of the midrib. Our tests showed that tomato leaves have AChE activity (578 pmol ATChI hydrolysed/mg protein/s) which is comparable to that present in nerves of lower animals. Naturally, the