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The Benefits of Ambiol® in Promoting Germination, Growth, and Drought Tolerance can be Passed on to Next-Generation Tomato Seedlings

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Abstract Seed preconditioning with Ambiol® has been shown to improve germination, growth, and drought tolerance in seedlings of many species. Attempts to understand the mode of action of Ambiol have found that seed preconditioning triggers several new proteins, which suggests that Ambiol-induced benefits may persist beyond seedlings and, perhaps, into the next generation. Seeds were preconditioned with 0 and 10 mg l^{-1} Ambiol to determine effects on germination, seedling growth, and yield of parent tomato plants. Seeds were collected from plants in each treatment and then sown to determine effects on germination and seedling growth in the next generation. Key parameters such as percent germination, leaf area, shoot mass, root mass, and photosynthesis were significantly improved in parents and in progeny. In addition, there was a 141% increase in tomato yield in preconditioned parents. It was concluded that Ambiol-induced benefits continue throughout plant development and into the next generation, potentially having significant horticultural and economic ramifications.

Keywords 5-Hydroxybenzimidazole · Water deficit · Drought stress · *Lycopersicum esculentum* · *Solanum lycopersicum* · Antioxidant · Seed preconditioning agent · Yield

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Introduction

Experiments with nitrogen-containing phenols, such as benzimidazoles, have demonstrated that antioxidants cause definite protective and regulative effects on the photosynthetic membrane in leaves when the root was under water stress (Li and Lin 1994). Ambiol® (2-methyl-4-[dimethylaminomethyl]-5-hydroxybenzimidazole) (Ambiol Inc., Toronto, ON) is a nitrogen-containing phenol that was synthesized in Russia using electrophilic substitution of 5-hydroxybenzimidazole with 4-aminomethyl derivatives (Smirnov and others 1985). The net result was seven different products, each possessing antioxidant properties and capable of promoting growth in various plants. Ambiol is considered the most effective and most studied derivative of 5-hydroxybenzimidazole (Smirnov and others 1985).

One of the earliest observed effects of Ambiol was on germination. Ambiol increased the percentage germination of seeds in specific half-sib black spruce families as well as in a bulk seed collection of black spruce (Borsos-Matovina 1997). Rajasekaran and others (2005) found that 0.1 mg l^{-1} Ambiol increased germination rates nearly fourfold in carrots, which confirmed previous reports of enhanced carrot germination in Ambiol-preconditioned seeds under limited soil moisture (Rajasekaran and others 2004). There was also some evidence of increased germination rate and percentage of tomato seeds after preconditioning with Ambiol (MacDonald 2006).

In general, Ambiol is considered an antioxidant (for example, Vichnevetskaia and Roy 1999; Voronina and others 2001), antistress agent (for example, Darlington and others .1996), and plant growth regulator (for example, Rajasekaran and Blake 1999, 2002). Growth of Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) seedlings was promoted after preconditioning with Ambiol. In fact, Ambiol-treated plants exceeded the control plants in height, length of needle-covered stem portion, needle weight, weight of needle-covered stem portion, length of roots, number of needles per plant, and length of needles (Vichnevetskaia and Roy 1999). Ambiol preconditioning increased root collar diameter in 4-month-old black spruce (*Picea mariana*) seedlings (Vichnevetskaia 1999). In a similar experiment by Borsos-Matovina (1997), Ambiol significantly increased root collar diameter, stem, root dry weight, and total biomass in 13-month-old black spruce, as well as root dry weight and total biomass of 13-month-old Jack Pine seedlings.

Perhaps the most interesting aspect of Ambiol is its ability to promote growth even under stressed conditions. Growth of drought-stressed soybean and rapeseed plants was stimulated by 25–45% after Ambiol preconditioning, yielding plants comparable in size to the fully irrigated controls (Darlington and others 1996). Ambiol also increased leaf area and shoot-to-root ratio under drought in soybean and rapeseed (Darlington and others 1996). Based on the work of Rajasekaran and Blake (2002), it has been shown that pretreating carrot seeds with 10 mg l^{-1} Ambiol can promote dry matter production under drought conditions by as much as 214% when compared to a drought control. Similar results have been found in tomato seedlings exposed to drought, where preconditioning with 10 mg l^{-1} Ambiol resulted in increased dry matter production, increased leaf area expansion, increased photosynthesis, and decreased membrane leakage in plants under drought (MacDonald and others 2008, 2009a, b). Ambiol also promoted growth under various other types of stress, including heat (Burlakova and others 1990; Colombo 1995), cold (Borsos-Matovina and Blake 2001), mineral deficiency (Budagovskaya and Guliaev 2004), and radiation (Vichnevetskaia and Roy 1999).

Although there is a plethora of evidence supporting Ambiol as a beneficial seed preconditioning agent, the regulatory pathway of Ambiol is still under investigation. It has been speculated that cytokinins and auxins are involved in the regulatory pathway of Ambiol, as Kirillova and others (2003) discovered elevated levels of indole acetic acid and zeatin after Ambiol foliar application. Conversely, abscisic acid (ABA) has essentially been ruled out as a hormone signal because foliar application of Ambiol decreased ABA by 50% (Kirillova and others 2003), and Ambiol preconditioning still induced drought tolerance in ABA-deficient mutant tomato seedlings and in the presence of an ABA inhibitor (MacDonald and others 2009b). Although more work is needed to understand the signal molecules involved in Ambiol-induced drought tolerance, it is thought that Ambiol preconditioning activates certain genes. Ambiol caused alterations to wheat DNA *in vitro* and *in vivo* (Voronina and others 2001) and altered protein expression both before and after germination (MacDonald and others 2009a). If Ambiol preconditioning activates certain genes that promote accelerated growth and drought tolerance, then how long might those effects last? Most studies to date have focused on the effects of Ambiol on seedlings, but a change in protein expression due to seed preconditioning with Ambiol suggests that Ambiol action is gene controlled, benefits may persist throughout plant development and maturation, and Ambiol's protective effects may be passed on to the next generation. Thus, the objective of this study was to determine the effects of Ambiol seed preconditioning throughout the development of a plant and passed on to the next generation. More specifically, this research examines the effect of Ambiol preconditioning on (1) seed germination, (2) unstressed and drought-stressed seedlings, (3) mature plant yield, (4) second-generation germination, and (5) second-generation unstressed and drought-stressed seedlings.

Materials and Methods

Seed Preconditioning

All seeds of tomato (*Solanum lycopersicum* L.) were preconditioned in the manner described by Rajasekaran and Blake (2002). 'Scotia' tomato seeds (Stokes Seeds Ltd, St. Catherines, ON) were preconditioned by placing 1 g of seeds into a 250-ml flask and soaking with 40 ml of either 0 or 10 mg l^{-1} Ambiol for 24 h (Ambiol Inc., Toronto, ON). Concentrations of Ambiol were chosen based on the work described by MacDonald and others (2008), who found that 10 mg l^{-1} was the most effective concentration to promote drought tolerance in tomato seedlings. All flasks were transferred to a G24 Environmental Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ) and incubated at 25°C and 150 rpm for 24 h. Contents of the flask were passed through a strainer and seeds were dried at 20°C.

Germination

Germination was evaluated by placing 100 seeds on moistened filter paper in a Petri dish stored at 20°C. This was replicated five times for each treatment. The Petri dishes were monitored daily to determine the number of germinated seeds, which was expressed as percent germination. Germination was monitored over 10 days, although no new seeds germinated after 6 days. In addition to percent germination, data were used to determine the time required for 50% germination (T_{50}) and mean time to germination (MTG). MTG was calculated using the following formula, described by Brenchley and Probert (1998), later modified by McDonald and Kwong (2005):

$$\mathrm{MTG} = \frac{(\sum T_i N_i)}{\sum N_i}$$

where T_i is each ith day and N_i is the number of germinated seeds on the ith day.

Seedling Growth

To evaluate the effect of Ambiol on tomato seedlings, a 2 × 2 factorial design was used. The first factor was Ambiol concentration: 0 versus 10 mg l^{-1}. The second factor was stress: well-watered versus a 6-day drought. Each treatment combination was replicated five times, thus twenty 15-cm pots were required. Each pot received 250 g of Promix BX (Premier Horticulture Inc., Red Hill, PA) and was initially fertilized with 250 ml 20-20-20 N:P:K fertilizer (Scotts-Sierra Horticultural Products Company, Maysville, OH). Each pot also received one seed, which had already undergone germination.

Pots were randomly arranged in an environment-controlled growth chamber with 25-16°C day-night temperatures, 50% relative humidity, 16-h photoperiod, and a 400-μmol m^{-2} s^{-1} light intensity. Each seedling was grown for 14 days under well-watered conditions, where they received 250 ml each day. On the 15th day, drought was imposed on half the plants by withholding water for 6 days. The other half continued to be well watered. Moisture content of the growth medium was measured throughout the experiment using a TDR 300 soil moisture probe (Spectrum Technologies, Plainfield, IL) at a depth of 12 cm. Moisture content before drought was recorded after the final watering on day 14. Moisture content after drought was recorded after 6 days of drought.

An LCA4 photosynthetic unit (ADC BioScientific Ltd., Herts, UK) was used to measure intrinsic net photosynthesis (P_n), instantaneous transpiration (T_l), and stomatal conductance (G_s) of the newest fully expanded leaf. Measurements started at 09:00 to minimize diurnal variation. Water use efficiency (WUE) was calculated as the ratio of photosynthesis to transpiration. After measurements with the LCA4, the newest fully expanded leaf was removed from a plant, placed on paper with a reference ruler, and photographed from directly overhead. The images of the leaves were uploaded to CIAS 2.0 Image Measurement software (Jandel Scientific, San Rafael, CA) to calculate leaf area and to properly adjust LCA4 measurements to account for actual leaf area as compared to leaf area in the LCA4 cuvette.

The chlorophyll index was measured using a Minolta SPAD 504 meter (Minolta, Ramsey, NJ). The Minolta meter was clamped onto the newest fully expanded leaf and the transmittance by the leaf at 650 and 940 nm was differentially absorbed by chlorophyll (Martinez and Guiamet 2004).

Membrane injury index (MII) was determined using the method described by Odlum and Blake (1996) and uses the percentage electrolyte leaking into solution to quantify membrane integrity. At the end of drought stress, test tubes were filled with 30 ml of distilled water and were allowed to adjust to room temperature (25°C). The electrical conductivity of the distilled water (EC_w) alone was measured using a CDM 2e Conductivity Meter (Bach-Simpson, London, ON). Afterward, the newest fully expanded leaf was separated from the stem and completely submerged in a test tube. The tubes were sealed and left at room temperature for 24 h. Initial conductivity (EC_0) was measured; this determined the amount of electrolytes leached into solution. Sealed tubes were then placed in a forced-air oven for 4 h at 90°C to kill tissues and then cooled to room temperature. Final conductivity measurements (EC_f) were taken after equilibrating to 25°C to determine maximum leakage. MII was then calculated using the formula from Odlum and Blake (1996):

$$\mathrm{MII} = \frac{EC_0 - EC_w}{EC_f - EC_w} \times 100$$

Plant height was recorded as the height from the cotyledons to the highest point of the tomato plant stem. Afterward, tomato seedlings were gently separated from the soil and then separated into roots and shoot. Each was placed in a forced-air oven for 24 h, and then the mass was recorded. The shoot and root dry masses were used to determine the shoot:root ratio.

Seed Extraction and Collection

A total of ten 15-cm pots were filled with 250 g of Promix BX and initially fertilized with 250 ml 20-20-20 N:P:K fertilizer. Half of the pots were sown with 0 mg l^{-1} Ambiol-preconditioned seeds, the other half with 10 mg l^{-1} Ambiol-preconditioned seeds. Each pot was transferred to an environment-controlled growth chamber with 25/16°C day/night temperatures, 50% relative humidity, 16-h photoperiod, and a 400-μmol m^{-2} s^{-1} light intensity. Seedlings were watered to field capacity (250 ml per day was sufficient). After 30 days, plants were transplanted into 30-cm pots and staked to keep the plants upright. Each pot held 1 kg of soil and was watered with 750 ml water and 250 ml of 2.4-g l^{-1} 20-20-20 N:P:K fertilizer daily.

Tomatoes were harvested from all plants at two points in time: after the first fruit cluster was ripe and after the second fruit cluster was ripe. Average tomato mass, yield (fruit/plant and g/plant), and seed count (seeds/plant) were recorded. Seeds were extracted from ripe tomatoes using fermentation. The fermented tomato pulp and seeds were passed through a strainer to remove all seeds and were rinsed several times with distilled water. Seeds were dried at 20°C overnight and then stored in sterile Petri dishes at 4°C for 60 days.

Next Generation

The stored seeds from the parent tomatoes did not undergo any further Ambiol preconditioning. Germination and seedling response of the next generation was conducted exactly as described above to determine if Ambiol-induced benefits were transferred to offspring.

Statistical Analysis

In evaluating germination responses (percent germination, T_{50}, and MTG) and response variables in mature plants (yield, tomato mass, and number of seeds), a two-sample *t* test was used (Minitab 15, Minitab Inc., State College, PA, USA). In all cases, assumption of normality was confirmed. Samples were compared at a 5% significance level. Statistical analysis of germination was conducted on the parents and then again on their progeny.

In evaluating seedling response variables (shoot and root mass, height, leaf area, MII, P_n, T_l, G_s, and WUE), the 2 × 2 factorial design was analyzed by SAS Proc GLM (SAS Institute, Cary, NC, USA). Statistical assumptions of normality, constant variance, and independence were all confirmed using Minitab 15. Multiple-means comparison was performed using least-squares means at a 5% significance level. Statistical analysis of seedlings was conducted on the parents and then again on their progeny. In the case of moisture content of the growing medium, no significant differences were detected between Ambiol-preconditioned seedlings before or after drought. Moisture content data before and after drought were pooled and expressed as an average moisture content.

Results

Germination of Ambiol-Preconditioned Parents

Preconditioning with 10 mg l^{-1} Ambiol significantly improved germination of tomato seedlings (Fig. 1, Table 1). In both 0- and 10-mg l^{-1} treatments, no germination was detected until day 4. However, 10 mg l^{-1} had a significantly higher percent germination for the remaining time (Fig. 1). At the end of germination, the 10-mg l^{-1} Ambiol treatment had increased percent germination by 12.4% and decreased T_{50} and MTG by 9.5% and 5.1%, respectively (Table 1).

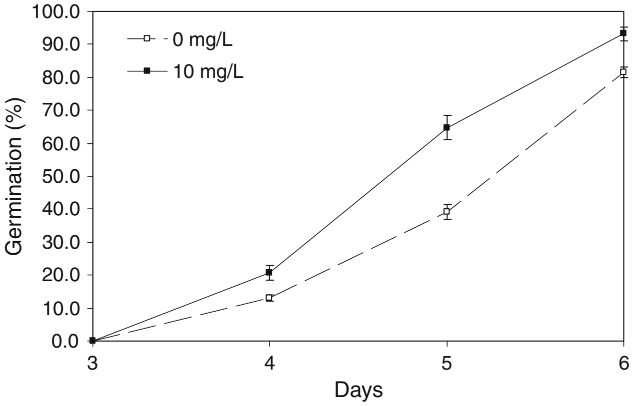

Fig. 1 Percent germination of 'Scotia' tomato seeds after preconditioning with 0 and 10 mg l^{-1} Ambiol®. *Error bars* represent the standard error calculated from five replications

Growth and Photosynthesis of Ambiol-Preconditioned Parents

Ambiol preconditioning had several significant effects on well-watered seedlings, which were maintained at 56% soil moisture. In terms of growth, preconditioning with 10 mg l^{-1} Ambiol significantly ($\alpha = 0.05$) increased height by 7.4%, shoot dry mass by 13.8%, root dry mass by 12.3%, and leaf area expansion by 28.1% compared to a well-watered control (Table 2). Regarding gas exchange measurements, P_n, T_l, and WUE increased by 67%, 28.6%, and 30.2%, respectively, compared to a well-watered control (Table 2). There were no significant differences in shoot:root ratio, MII, or G_s.

In the droughted treatments, soil moisture dropped to an average of 3%. There were no differences in soil moisture between 0- and 10-mg l^{-1} preconditioned seedlings. There were, however, significant ($\alpha = 0.05$) differences in several other parameters observed. Those droughted seedlings preconditioned with 10 mg l^{-1} Ambiol experienced a 16.2% increase in height, 24.1% increase in root mass, 81.8% increase in shoot mass, 47.1% increase in shoot:root ratio, 36.3% increase in leaf area expansion, 34.5% increase in P_n, and 26% increase in WUE when compared to droughted controls (Table 2). There were no differences detected in MII, T_l, or G_s.

Yield and Seed Production of Parents

The first clusters of flowers were noticed on the 10-mg l^{-1} preconditioned plants 45 days after emergence. Flowers were noticed on the 0-mg l^{-1} preconditioned plants 50 days after emergence. Preconditioning with 10 mg l^{-1} Ambiol had a significant effect ($\alpha = 0.05$) on all recorded parameters from the mature plants. There was an increase of 65.1% in number of tomatoes on each plant and an

Table 1 Changes in germination in 'Scotia' tomato seeds preconditioned with Ambiol®

Treatment	Germination (%)	T_{50} (days)	MTG (days)
Control	81.6 ± 1.5	5.3 ± 0.1	5.36 ± 0.03
Ambiol	93.0 ± 2.0	4.8 ± 0.1	5.09 ± 0.06
95% CI	(−14.0, −8.8)	(0.36, 0.61)	(0.20, 0.33)
P value	0.0001	0.0002	0.0002

Data are expressed as mean ± standard error as calculated from five replications

T_{50} time required for 50% of seeds to germinate, *MTG* mean time until germination; Control = 0 mg l^{-1}; Ambiol = 10 mg l^{-1}

P values and confidence intervals were calculated from a two-sample *t* test

Table 2 Effects of Ambiol® preconditioning on well-watered and drought-stressed 'Scotia' tomato seedlings

Response	Well-watered		Drought	
	Control	Ambiol	Control	Ambiol
Height (cm)	9.73^{b}	10.45^{a}	9.24^{b}	10.74^{a}
Root DM (g)	1.135^{a}	1.275^{a}	0.996^{c}	1.236^{a}
Shoot DM (g)	1.683^{c}	1.915^{b}	0.848^{c}	1.542^{b}
Shoot:root	1.48^{a}	1.50^{a}	0.85^{c}	1.25^{b}
Leaf area (cm^2)	6.40^{c}	8.20^{ab}	7.00^{b}	9.54^{a}
MII (%)	11.2^{a}	11.9^{a}	17.9^{b}	15.9^{b}
Pn (μmol m^{-2} s^{-1})	1.819^{b}	3.037^{a}	0.834^{d}	1.122^{c}
Tl (mmol m^{-2} s^{-1})	1.405^{b}	1.807^{a}	0.483^{c}	0.514^{c}
Gs (mol m^{-2} s^{-1})	0.256^{a}	0.271^{a}	0.0290^{b}	0.0296^{b}
WUE (Pn/Tl)	1.29^{c}	1.68^{b}	1.73^{b}	2.18^{a}

Control = 0 mg l^{-1}; Ambiol = 10 mg l^{-1}

Data are expressed as the mean of five replications for each treatment combination. Different letters indicate a significant difference at the 5% significance level

increase of 44.5% in tomato mass. As result, there was a significant 141% increase in overall tomato yield. In addition to effects on yield, there was also a 143% increase in seeds/tomato (Table 3).

Germination of the First-Generation Progeny

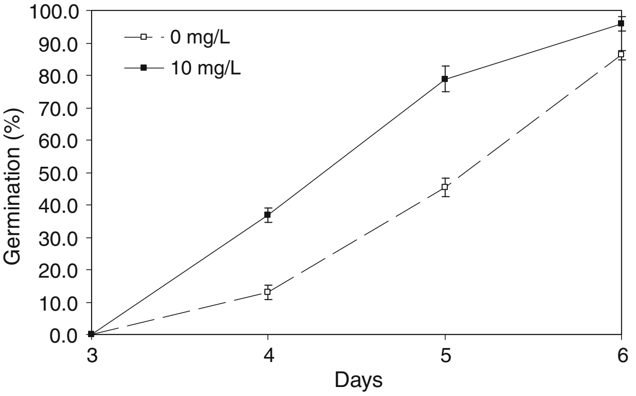

Fig. 2 Percent germination of 'Scotia' tomato seeds harvested from Ambiol-preconditioned parents. *Error bars* represent the standard error calculated from five replications

Germination was significantly enhanced in seeds harvested from parent tomato plants that were preconditioned with 10 mg l^{-1} Ambiol. As observed with parents, no germination was detected until day 4 in either treatment. However, once germination started, seeds from the Ambiol-preconditioned plants had a higher germination rate (Fig. 2). At the end of germination, seeds from the 10 mg l^{-1} Ambiol-preconditioned parent had increased percent germination by 9.6% and decreased T_{50} and MTG by 15.4% and 10.0%, respectively (Table 4).

Growth and Photosynthesis of the First-Generation Progeny

The difference in the growth of seedlings grown from seeds harvested from Ambiol-preconditioned plants compared to those grown from control plants was immediately obvious

Table 3 Changes in yield and seed production in 'Scotia' Tomato plants preconditioned with Ambiol®

Treatment	No. tomatoes	Mass/tomato (g)	Yield (g/plant)	Seeds/tomato
Control	8.6 ± 3.6	18.18 ± 2.96	158.9 ± 84.9	28.2 ± 8.1
Ambiol	14.2 ± 4.0	26.27 ± 1.20	382.5 ± 105.6	68.5 ± 2.2
95% CI	(−11.2, −0.1)	(−12.08, −5.50)	(−363.4, −83.9)	(−45.3, −28.0)
P value	0.0485	0.0003	0.0061	<0.0001

Control = 0 mg l^{-1}; Ambiol = 10 mg l^{-1}

Data are expressed as mean ± standard error as calculated from five replications. The *P* values and confidence intervals were calculated from a two-sample *t* test

Table 4 Changes in germination of 'Scotia' tomato seeds harvested from Ambiol-preconditioned parents

Treatment	Germination (%)	T_{50} (days)	MTG (days)
Control	86.2 ± 1.5	5.2 ± 0.1	5.32 ± 0.05
Ambiol	95.8 ± 2.3	4.4 ± 0.1	4.79 ± 0.04
95% CI	(−12.4, −6.8)	(0.72, 0.92)	(0.46, 0.59)
p value	0.0005	<0.0001	<0.0001

T_{50} time required for 50% of seeds to germinate, *MTG* mean time until germination; Control = 0 mg l^{-1}; Ambiol = 10 mg l^{-1}

Data are expressed as mean ± standard error as calculated from five replications. The *P* values and confidence intervals were calculated from a two-sample *t* test

Fig. 3 Differences in growth of unstressed 'Scotia' tomato seedlings grown from seeds harvested from Ambiol-preconditioned parents: (*left*) seedling from the 10 mg l^{-1} Ambiol-preconditioned parent; (*right*) seedling from the 0 mg l^{-1} (control) Ambiol-preconditioned parent

(Fig. 3). All plants were maintained at an average of 51% soil moisture with 250 ml of water daily, but there was a 30.8% increase in plant height, 90.4% increase in root mass, 127.0% increase in shoot mass, and 30.3% increase in leaf area expansion in Ambiol-preconditioned seedlings when compared to well-watered controls. In addition, there was a 21.9% increase in P_n, 20.7% increase in T_l, and 38.0% increase in G_s compared to those parameters of well-watered controls (Table 5).

When exposed to drought, soil moisture declined to 5%. There was no difference in soil moisture between 0- and 10-mg l^{-1} Ambiol treatments. However, the first-generation seedlings under drought stress that were grown from Ambiol-preconditioned plants still had a 26.9% increase in height, 79.3% increase in root mass, 91.2% increase in shoot mass, 30.3% increase in leaf area, 235% increase in P_n, 69.6% increase in T_l, 183% increase in G_s, and 24.1% increase in WUE when compared to the non-Ambiol-preconditioned seedlings under drought (Table 5).

Table 5 Differences in growth and selected gas exchange parameters in well-watered and drought-stressed 'Scotia' tomato seedlings grown from Ambiol-preconditioned parents

Response	Well-watered		Drought	
	Control	Ambiol®	Control	Ambiol®
Height (cm)	9.08^{b}	11.88^{a}	8.70^{b}	11.04^{a}
Root DM (g)	0.878^{b}	1.672^{a}	1.032^{b}	1.850^{a}
Shoot DM (g)	1.214^{c}	2.754^{a}	1.048^{c}	2.004^{b}
Shoot:root	1.38^{b}	1.65^{a}	1.02^{c}	1.08^{c}
Leaf area (cm^2)	8.49^{b}	9.44^{a}	6.61^{c}	8.61^{b}
MII (%)	12.5^{a}	14.0^{a}	14.1^{a}	14.4^{a}
Pn (μmol m^{-2} s^{-1})	0.575^{d}	3.370^{a}	0.575^{d}	1.927^{c}
Tl (mmol m^{-2} s^{-1})	0.257^{d}	2.293^{a}	0.257^{d}	0.693^{c}
Gs (mol m^{-2} s^{-1})	0.0238^{d}	0.334^{a}	0.0238^{d}	0.0674^{c}
WUE (Pn/Tl)	2.24^{b}	1.47^{c}	2.24^{b}	2.78^{a}

Control = 0 mg l^{-1}; Ambiol = 10 mg l^{-1}

Data are expressed as the mean of five replications for each treatment combination. Different letters indicate a significant difference at the 5% significance level

Discussion

Benefits of Ambiol-Preconditioning on Parents

The overall benefits of Ambiol preconditioning on tomato seeds and seedlings were similar to those described in our previous research. Ambiol was effective at increasing both the germination rate and total percentage of germinated seeds, which was first suggested in tomato based on observations by MacDonald (2006). The ability of Ambiol to encourage germination has also been noted in other species such as black spruce and carrot seedlings (Borsos-Matovina 1997; Rajasekaran and others 2005). Ambiol-preconditioned seedlings grown under well-watered and droughted conditions had increased shoot and root growth, leaf area expansion, and photosynthesis, which was consistent with a number of other studies. For example, MacDonald and others (2008, 2009a, b) reported increased growth and photosynthesis in both stressed and unstressed tomato seedlings. In addition, increased growth due to Ambiol preconditioning was reported in several other species, including soybean (Darlington and others 1996), rapeseed (Darlington and others 1996), potato (Evsyunina and others 2002), and carrot (Rajasekaran and Blake 2002).

Potential Mechanisms for Ambiol

The exact physiological mechanism of Ambiol has been difficult to determine, although certain aspects have been well studied. It has been established that Ambiol invokes certain proteins within a seed immediately after preconditioning (MacDonald and others 2009a), which ultimately results in profound improvements to growth, membrane stability, and photosynthesis. The events between activated proteins and improved growth are not completely understood. In the past it has been speculated that Ambiol acts as an antioxidant and/or antitranspirant (Darlington and others 1996; Rajasekaran and Blake 2002), but evidence to support those claims is contradictory. For example, it has been shown that Ambiol has considerable antioxidant potential *in vitro* (Voronina and others 2001) and has improved membrane stability in several conifer species and carrot seedlings under drought (Rajasekaran and Blake 1999; Borsos-Matovina and Blake 2001; Islam and others 2003). Similarly, Ambiol preconditioning has been found to significantly reduce transpiration compared to control seedlings (Rajasekaran and Blake 2002). However, there were no improvements to membrane protection in this study and limited benefits from previous studies with tomato seedlings (MacDonald and others 2008, 2009a). Also, in all previous studies about Ambiol-preconditioned tomato seedlings, transpiration actually increased compared to that of control seedlings (MacDonald and others 2008, 2009a, b). Yet seedlings always experience increased growth under drought.

One purpose of measuring the membrane injury index and transpiration is to evaluate the amount of stress imposed on a plant (Odlum and Blake 1996). Under drought stress, a seedling would be expected to have higher membrane leakage and lower transpiration. Perhaps it should be considered that improved membrane stability and higher transpiration may not be the *cause* of increased drought tolerance in seedlings but rather the *consequences* of increased drought tolerance. If a seedling is better able to withstand drought, it stands to reason that there would be less damage to biological membranes and there would be no reason to close stomata as early. If Ambiol is not functioning as an antioxidant or antitranspirant, how does Ambiol confer benefits to seedlings?

It was once noted that a key benefit of Ambiol was modification of WUE (Darlington and others 1996), later speculated to be caused by enhancements to root growth (Rajasekaran and Blake 2002). Improvements to root growth under drought were detected in this study as well as in all other studies involving Ambiol-preconditioned tomato seedlings (MacDonald and others 2008, 2009a, b). Due to the substantial growth-regulating properties of Ambiol, Kirillova and others (2003) hypothesized that there might be Ambiol-induced changes in certain phytohormones. Concentrations of ABA, the auxin indoleacetic acid (IAA), and the cytokinin zeatin were measured after treatment with Ambiol. It was found that Ambiol treatment decreased ABA concentrations by 50%. However, there was a fivefold increase in IAA and a threefold increase in zeatin. Increases in cytokinins and auxins are known to encourage shoot and root growth (Zahir and others 2001; Teale and others 2006). Further study of Ambiol-induced hormonal changes is required, but current evidence points more to a role in plant growth regulation than a role as antioxidant or antitranspirant.

One limitation of many studies on Ambiol is that it is difficult to distinguish whether Ambiol functions directly as an antistress agent or whether improved stress tolerance is a secondary effect due to the growth-regulating properties of Ambiol. In other words, the decrease in the time required for emergence of seeds treated with Ambiol could enable plants to become established more rapidly and therefore enhance their viability. In the case of conifers, there was no significant difference detected between Ambiol-preconditioned and control unstressed seedlings (Islam and others 2003). However, there was significant improvement in unstressed tomato seedling shoot growth after Ambiol preconditioning, similar to that seen in past experiments (Rajasekaran and Blake 2002; MacDonald and others 2008). Observed increases in shoot mass and germination in the absence of growth would suggest that stress tolerance is a secondary effect, but it should be noted that in this and past studies the enhanced root growth did not occur until imposition of drought (Rajasekaran and Blake 2002; MacDonald and others 2008). Thus, at least a portion of Ambiol-induced effects directly promote drought tolerance.

Benefits of Ambiol on Plant Yield

This experiment was the first one known to look at the effects of Ambiol on plant yield. Naturally, further experiments are required to test the effect on several crops, but these results reveal a great potential for Ambiol to increase yield of horticulture crops. The yield of ‘Scotia’ tomato plants was more than doubled as a result of Ambiol preconditioning, attributed to significant increases in the number of tomatoes on each plant and average tomato size. Likewise, Ambiol offers potential in seed production because it increased seed content compared to the untreated plants.

It should be emphasized that the increase in tomato yield is not due to a carry over effect from accelerated germination and, potentially, accelerated plant development. Unlike the analysis of tomato seedlings, which were all conducted on the same day, tomatoes were harvested when tomato plants were at the same developmental stage, when the first and second sets of fruit were ripe. The decision to harvest fruit at the same developmental stage was made when it was noted Ambiol-preconditioned plants developed flowers nearly a week before the controls. As a result, increases in tomato number, size, and seed count must be a result of Ambiol preconditioning of the seed.

Because Ambiol is a synthetic compound, economic and food chemistry studies would have to follow to ensure viability to the producer and safety to the consumer. However, it is worth noting that several naturally occurring antioxidants were found to precondition tomato seedlings in a manner similar to Ambiol (MacDonald and others 2009a). Ascorbic acid, β-carotene, and lycopene were all found to have growth-regulating and antistress properties as seed preconditioning agents. Ascorbic acid and β-carotene also altered protein expression before and after germination (MacDonald and others 2009a). Although it remains unknown how long the effects of the aforementioned natural antioxidants would persist, their striking similarity to Ambiol in function and protein expression lends to the idea that they could provide a natural method for transgeneration carryover of drought tolerance and to boost yield.

Benefits of Ambiol to First-Generation Progeny

Perhaps the most remarkable results of this study can be found in seeds and seedlings grown from seeds collected from preconditioned parent plants. Those seeds collected from Ambiol-preconditioned parents had an increased percent germination combined with decreased germination times, a trend similar to that observed in the preconditioned parents. In addition, seedlings grown from Ambiol-preconditioned parents had increased growth, where shoot and root growth nearly doubled the controls. Parent-generation plants were never exposed to drought, therefore, the effects observed in next-generation plants must be a result of Ambiol preconditioning. One early hypothesis about Ambiol was that more vigorous growth of pine seedlings may be attributed to the fact that Ambiol evoked dormant genes (Vichnevetskaia and others 1992). Recent experiments by Briscoe (2005) and MacDonald and others (2009a) confirmed that preconditioning altered the protein expression within a seed, which may indicate inhibition or activation of dormant genes. The fact that Ambiol-induced effects are evident in mature plants and next-generation seedlings would suggest that there is a level of gene control involved.

Commercial Potential for Ambiol in Horticulture

Overall, the data suggest that the effects of Ambiol preconditioning are observed as early as germination, persist throughout plant development, and are eventually transferred to the next generation, although further studies would be needed to understand the extent of persistence and consistency in response for different species. Based on improvements in germination, growth, stress tolerance, and yield, there are great commercial applications for Ambiol in horticulture. In addition, these findings support the conclusions found by MacDonald and others (2009a) regarding changes in protein expression.

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