Is Endogenous Ethylene Production Associated with Increased Shoot Number in Gentian Cultured In Vitro? Developing a Method for Ethylene Measurement at Nano Level within a Gas-exchangeable In Vitro System

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Abstract. We investigated the possibility of either exogenous ethylene or endogenous ethylene production having an association with the increase in shoot number when nodal explants of *Gentiana* spp. ‘Little Pinkie’ were cultured in an in vitro medium supplemented with ethephon (10 mg·L⁻¹). For the first time within an in vitro system, we report the application of laser ethylene detector technology, and optimization of the methodology to quantify concentrations of ethylene (in the part-per-billion range) released from ethephon decomposition within the atmosphere of gas-exchangeable culture vessels including nodal explants. Compared with continuous (continuous measurements on the same replicate of vessels) and repeated (sampling same replicate of vessels every 48 hours) sampling methodologies, the nonrepeated (sampling fresh replicate of vessels every 48 hours) method of measurement of ethylene concentration was more representative of the actual condition within vessels. Although no prior published data exist showing the positive or negative effect of gaseous ethylene in the headspace of culture vessels on bud outgrowth in gentian, our study shows gaseous ethylene in the headspace of culture vessels was not effective in increasing shoot formation in gentian explants cultured in vitro, whereas ethephon supplementation in agar was effective. Plant material in culture vessels did not have a significant effect on ethylene production regardless of the presence or absence of ethephon. Therefore, although ethephon supplementation in the medium produced gaseous ethylene in the headspace, it was unlikely to cause endogenous ethylene production in explants, but it did trigger shoot formation in ‘Little Pinkie’, perhaps through decomposition to ethylene within the explant tissue, enhancing the internal ethylene level possibly at a locally high concentration.

The number of shoots per propagule influences shoot predisposition to form a high-quality potted plant, and within an in vitro propagation system it directly determines the yield of nodal explants that can be used either for routine propagation to build up numbers or deflasked for production scheduling. In explants of *Gentiana* ‘Little Pinkie’ (naturally highly branched), shoot formation increased as a result of ethephon

supplementation in vitro whereas shoot formation did not increase in explants of *Gentiana* ‘Showtime Diva’ (naturally less branched) (Keshavarzi et al., 2014). Regardless of the cultivar and whether grown in vivo or in vitro, information on growth and shoot branching in gentian is very limited. Also, whether the difference between cultivars in their shoot branching was associated with the

endogenous ethylene production needed to be explored. Therefore, our study was carried out to understand the basis of the differential response between cultivars of gentian to ethephon/ethylene that may lead to strategies to increase shoot formation in low-branching cultivars of gentian.

We hypothesized that ethephon exerts its effect on increasing shoot formation in ‘Little Pinkie’ through its decomposition to ethylene in the headspace of culture vessels, and thus investigated the putative effects of ethephon and ethylene on the plants in a culture vessel capable of gas exchange. Previous studies on the presence of ethylene in vitro have merely described the negative/positive effects of ethylene/ethephon on growth variables of plants (Harbage and Stimart, 1996; Kerbauy and Colli, 1997), in contrast to detailing an ethephon–ethylene relationship within an unsealed culture vessel used for in vitro culture. The culture vessels used in our study needed to be unsealed to allow for gas exchange during plant growth in vitro. When ethephon was added to the medium, the concentration of ethylene in the headspace of the vessel to which explants were exposed became a technical challenge to quantify. Hence, to investigate the current hypothesis in the first experiment we quantified the dynamics of ethylene concentration in the headspace of culture vessels without explants using laser ethylene detector technology. Our earlier research reported ethylene concentration at 48-h intervals using a method that removed accumulated ethylene in the headspace and replaced it with ethylene-free air at each measurement time (Keshavarzi et al., 2014). Within the current research, continuous measurement monitored ethylene concentration in real time, which relied on a continuous stream of ethylene-free gas passing over the headspace of culture vessels (Cristescu et al., 2008; Forni et al., 2012; Milenaar et al., 2009; van den Dungen et al., 2011). With both these methods, the actual concentration of ethylene in the headspace of culture vessels with explants could be less than the equilibrium concentration in empty culture vessels. Therefore, our report here, for the first time within an in vitro system, includes evaluation of measurement methods using laser ethylene detector technology. We optimized the method that best quantifies the actual concentration of ethylene in the headspace of gas-exchangeable culture vessels.

With the ethylene measurement methodology established, knowing that ethephon decomposes to ethylene (Yang, 1969), we assessed plant responses to the direct application of gaseous ethylene to test the hypothesis that ethylene in the headspace was the active compound. An experiment was carried out to address the question of whether biological activity (increase in shoot number) required ethephon to enter the explant tissue directly, or whether ethylene released from ethephon in the headspace of the same culture vessel was effective on its own. The possibility of any difference in the concentration of ethylene produced within vessels with ethephon-

amended medium containing two different gentian cultivars that respond differently to ethephon as a shoot formation stimulant was also investigated in another experiment.

Materials and Methods

All base media were modified from Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) free of plant growth regulators, comprising MS macro salts at half strength, MS micronutrient salts, Linsmaier and Skoog vitamins (Linsmaier and Skoog, 1965), 7.5 g·L⁻¹ agar, and 3% (87.6 mM) sucrose (Morgan and Bicknell, 1997). Ethephon (Ethrel; 480 g·L⁻¹ chlorethephon; Bayer Crop Science, Auckland, New Zealand) was added to selected media via filter sterilization (0.2 µm Minisart filter; Sartorius Stedim Biotech, Germany) at 10 mg·L⁻¹ if required for the experiment. Fifty milliliters of medium was put in each plastic culture vessel (250-mL volume; Alto Packaging, Hamilton, New Zealand) with snap-on lids to allow gas exchange. Unless stated otherwise, the same culture vessels were used for all experiments. Plant material was obtained from *in vitro* shoots of the gentian cultivars Showtime Diva and/or Little Pinkie, with each cultivar being put through six cycles of subculturing in the medium free of plant growth regulators. *In vitro* shoots were cut into single nodes of known position and were cultured *in vitro* at 25 ± 1 °C, with a photosynthetic photon flux density of 30 ± 5 µmol·m⁻²·s⁻¹ and a 16-h photoperiod (Keshavarzi et al., 2016).

Optimization of methodology, ethylene concentration, and rate of production measurement. For quantification of ethylene concentration and rate of ethylene production, two methods were evaluated. The first was a continuous flow method, in which three culture vessels containing base medium supplemented with ethephon were sealed with clear glue using a hot-glue gun and were connected to a commercial laser-based ethylene detector [type ETD-300 (ETD); Sensor Sense B.V., Nijmegen, the Netherlands] (Gwanpua et al., 2018), with the first measurements conducted within 2 h of preparing the media. The ETD provided a very slow continuous flow of humidified carrier gas [relative humidity (RH),

≈95%] through each vessel (1 L·h⁻¹). Data handling software (Valve controller 1.4.1, Sensor Sense) provided hourly average values for the amount of ethylene that had been quantified every 6 s, representing the rate of ethylene release from the medium. The second method was the sampling method, in which the internal atmosphere of each unsealed vessel with a rubber septum on its lid was sampled sequentially at a flow rate of 5 L·h⁻¹ and was sent to the ETD for measurement. The first measurements were taken 4 h after adding ethephon to the medium and closing the lid, followed by subsequent measurements at 48-h intervals, for a period of 2 weeks. Data handling software was used to calculate the entire quantity of ethylene in the vessel at the time of measurement, as well as the rate of ethylene production, to be compared with that measured by the continuous flow method. During repeated measurements, the same three replicates of culture vessel were used for every measurement over the 2-week period of observation; for the non-repeated measurement, culture vessels were discarded after each measurement and therefore three fresh replicates were used at each measurement time. The experiment was based on a completely randomized design.

Aerial exposure of the explants to ethylene gas. Culture vessels each containing eight nodal explants of 'Little Pinkie' (responsive cultivar to the application of ethephon) cultured in base medium were exposed to a flow rate of 1 L·h⁻¹ of externally supplied humidified (RH, ≈95%) and filtered (0.2-µm membrane filter, MINISART; Global Scientific Company, Germany) air, flushing through the culture vessels continuously. Treatments comprised a factorial combination of with or without ethylene (either 0 or 60 nL·L⁻¹ ethylene (BOC Ltd., Christchurch, New Zealand) over nine durations of exposure (0, 4, 24, or 48 h; or 1, 2, 4, 6, or 8 weeks), and were applied on three replications of vessels. The number of shoots was recorded for individual explants after 8 weeks of growth.

Aerial vs. direct contact of explants to ethephon/ethylene. To allow interpretation of the shoot formation response to the form of ethephon/ethylene exposure, a different culture system from that used in previous experiments was used. In this culture system, a sterilized plastic petri dish (diameter, 55.6 mm) containing nodal explants cultured in the base medium (with the same nutritional conditions as in the culture vessel) was suspended inside the culture vessel containing the base medium supplemented with either 0 or 10 mg·L⁻¹ ethephon. Doing this provided the opportunity of exposing only aerial parts of the nodal explants cultured in the petri dish to ethylene released from ethephon in vessels without actually being in direct contact with ethephon-containing medium, unless included within the petri dish. Therefore, there were four treatments: 1) explants cultured in a petri dish containing ethephon-free medium suspended in a culture vessel containing ethephon-free medium, 2) explants cultured in a petri dish containing ethephon-free medium suspended within the main culture

vessel containing 10 mg·L⁻¹ ethephon in the basal medium, 3) explants cultured directly in the culture vessel containing ethephon-free medium, and 4) explants cultured directly in the culture vessel containing 10 mg·L⁻¹ ethephon in the basal medium. Treatments were type of exposure to ethylene/ethephon (i.e., from medium, from atmosphere, or no exposure). Eight culture vessels or petri dishes were used as individual replicates, with four single nodal explants as experimental units within each container. After 8 weeks of culture, the numbers of shoots per explant were recorded.

Ethylene production by explants as affected by ethephon *in vitro*. Last, to assess the effect of ethephon on ethylene production by explants *in vitro*, ethylene concentration was measured in culture vessels containing a 2 × 2 factorial arrangement of cultivar ('Little Pinkie' or 'Showtime Diva') and ethephon concentration (0 mg·L⁻¹ or 10 mg·L⁻¹), plus controls (no explants) using the repeated method. Three culture vessels, each containing eight nodal explants, were used as replicates for each treatment.

Data analysis. For all experiments, data were analyzed using the general linear model in Genstat (Genstat, version 17; VSNi Ltd., Hemel Hempstead, UK). Data transformation was carried out if required for normality, and post hoc mean separation used Fisher's protected least significant difference test at $P \leq 0.05$.

Results

Optimization of methodology, ethylene concentration, and rate of production measurement. The concentrations of ethylene detected in the vessel using both repeated and nonrepeated sampling methods followed similar patterns of ethylene release over time (Fig. 1). The concentration fluctuated between 40 and 80 nL·L⁻¹ for the entire measurement time, reaching a peak at day 2 for both methods (not significantly different), followed by a decrease that was considered negligible (non-repeated: slope = 0.00, $P = 0.982$; repeated: slope = -0.16, $P = 0.233$).

The rates of ethylene production using both methods were not different from each other for the first 4 d, but then decreased significantly after day 4 only when the sampling method was applied (Fig. 2). The average rate of ethylene production measured by the continuous method was 1.3 times greater than when it was measured using the sampling method ($P \leq 0.05$).

Aerial exposure of the explants to ethylene gas. Atmospheric exposure of *in vitro* explants of 'Little Pinkie' to 60 nL·L⁻¹ ethylene gas in culture vessels, for any of the time periods tested (i.e., from 4 h to 8 weeks), did not affect significantly the number of shoots formed on nodal explants compared with the control ($P = 0.176$, Fig. 3). Numbers for all treatments averaged two shoots per explant (Fig. 3).

Aerial vs. direct contact of explants to ethephon/ethylene. Nodal explants grown in culture vessels including ethephon in their medium (treatment 4) produced approximately twice as many shoots as the explants

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Fig. 1. Concentration of ethylene in the headspace of unsealed culture vessels, measured by non-repeated (solid line, every vessel was sampled only once at one time point) or repeated (dotted line, sampling method with every culture vessel sampled at every time point), beginning 4 h after adding ethephon. For clarity, vertical lines only represent $\pm$ SE bars of the mean values. Fisher's protected least significant difference test was used for mean comparison at $P \leq 0.05$ ($n = 3$).

of all other treatments ($P \leq 0.001$) (Fig. 4). Aerial exposure of the explants to ethylene only, without direct contact of the tissue with ethephon in the medium (treatment 2), did not result in any significant change in shoot formation in comparison with explants cultured without ethephon in the medium or ethylene in the atmosphere (treatments 3 and 4).

Ethylene production by explants as affected by ethephon in vitro. In the presence of ethephon but without explants, the atmosphere inside the vessel contained an average of 59 ± 5.5 nL.L⁻¹ ethylene, which was significantly greater than in vessels without either ethephon or explants (i.e., 0.4 ± 0.16 nL.L⁻¹; $P \leq 0.05$; Fig. 5A). Although not statistically significant, the concentration of

ethylene peaked at about 48 h in the presence of ethephon. In the absence of ethephon, regardless of the presence or absence of explants in vessels (Fig. 5B), the ethylene concentration remained about constant and extremely low (< 2 nL.L⁻¹).

Inclusion of explants in the absence of ethephon resulted in an increase (up to 2 ± 0.2 nL.L⁻¹) in the concentration of ethylene in vessels compared with that in vessels without any explants, which was statistically significant only at the first measurement time (4 h from the commencement of the experiment; $P \leq 0.05$). The concentration of ethylene in vessels with and without explants, in the absence of ethephon, was not statistically significantly different for the rest of the measurement times. In vessels without explants,

Fig. 2. Rate of ethylene production within culture vessels containing 10 mg.L⁻¹ ethephon as measured by ethylene detector (type ETD-300) using either the sampling (dotted line) or the continuous (solid line) method, with humidified airflow. For the continuous treatment, data points represent the 2-d average; for the sampling method, treatment data were derived from only one time point every 2 d. Vertical lines represent $\pm$ SEM. Fisher's protected least significant difference test was used for means comparison. Means followed by different letters are significantly different at $P \leq 0.05$ ($n = 3$).

there was no ethylene detected at 4 h, whereas it was up to 2 nL.L⁻¹ in vessels containing explants, depending on the cultivar. In other words, vessels containing 'Little Pinkie' had twice as much ethylene as those containing 'Showtime Diva' ($P \leq 0.05$). The ethylene concentration after the first 4 h remained unchanged for all treatments at an amount that did not differ statistically throughout the rest of the experiment ($P \leq 0.05$; Fig. 5A).

Discussion

Despite an earlier report of a potential correlation between the increases in gaseous ethylene in the headspace of culture vessels supplemented with ethephon and bud outgrowth of gentian (Keshavarzi et al., 2014), our study shows gaseous ethylene in the headspace was not effective on bud outgrowth (Fig. 4). This finding was reaffirmed following exogenously supplied gaseous ethylene rather than ethephon derived (Fig. 3). In contrast, however, direct contact of the explants with ethephon in the medium increased shoot formation (Fig. 4). Except for our report, no previous published data were found on the effect of gaseous ethylene in the headspace of in vitro vessels on bud outgrowth in gentian. However, with in vitro callus of *Daucus carota* L., exogenously supplied ethylene at a concentration of 5 μ L.L⁻¹ over a 4-week culture as well as the ethylene released from ethephon was not effective on embryogenesis, whereas direct contact of the callus with ethephon in the medium reduced it (Tisserat and Murashige, 1977). As did Tisserat and Murashige (1977), we conclude aerial contact to ethylene from the headspace is not the mechanism to create the final concentration of ethylene required at the active site that regulates the change in explant physiology. Direct exposure of the explants to ethephon from the medium, and then decomposing to ethylene in the tissue, could potentially generate a greater effective internal concentration of ethylene in the explants (Johnston et al., 2002), which could be responsible for generating the increased shoot formation response.

As reported by Nir and Lavee (1981), ethephon breaks down rapidly during the first hours after application that would produce a pulse of high concentration of ethylene in the tissue. Similarly, in our research, a pulse of 80 nL.L⁻¹ internal concentration of ethylene was quantified at day 2 (Fig. 1) and could have been responsible for the increase in shoot formation, compared with no response to the aerial continuous application of ethylene at 60 nL.L⁻¹ for 8 weeks. Hence, a brief pulse of internal ethylene may be an avenue to follow for further research. As evidence for the difference between a pulse and a continuous application of other plant growth regulators on growth variables of explants in vitro, explants exposed to a pulse treatment of benzyl adenine enhanced growth about three times more than longer term application (Arigita et al., 2005). Such a difference between short-term and long-term application of a plant growth regulator could also be related to the

Fig. 3. Number of secondary shoots developed per explant of gentian 'Little Pinkie' exposed to a flow rate of 1 L·h⁻¹ externally supplied air, over nine durations of exposure (0, 4, 24, or 48 h; or 1, 2, 4, 6, or 8 weeks). Numbers of shoots were recorded for individual explants after 8 weeks of growth. For means separation, Fisher's unprotected least significant difference was applied to square root-transformed data; back-transformed values are presented. Vertical lines represent $\pm$ SEM ($P \leq 0.05$, $n = 3$ vessels).

sensitivity of the tissue at the time of the application. Explant tissue was reported to show the most sensitivity for rooting to a 24-h ethylene pulse 2 to 3 d after cuttings were taken (Robbins et al., 1985). Such differences could also explain why aerial application of ethylene to explants did not affect shoot formation whereas application of ethephon in the medium did. Because the aerial exposure to gaseous ethylene for various durations did not affect shoot number in explants, the critical duration of ethephon/ethylene exposure on increasing shoot formation remains to be investigated.

An argument for why application of gaseous ethylene in the headspace was not effective on bud outgrowth in 'Little Pinkie' could be that a concentration of gaseous ethylene greater than 60 nL·L⁻¹ might be needed to make a greater effective internal concentration of ethylene in the explants to generate

the response. In this case, to generate the concentration of gaseous ethylene greater than 60 nL·L⁻¹ in the headspace, a concentration greater than 10 μ L·L⁻¹ ethephon in the medium might be needed. However, application of a concentration greater than 10 μ L·L⁻¹ ethephon in the medium within a unsealed in vitro system may not necessarily result in a concentration of gaseous ethylene greater than 60 nL·L⁻¹ as a result of the constant leakage from vessels. Also, presumably some of the released molecules of ethylene in the headspace could always be returned to the medium if they could not leave the vessel [Boyle, 1662; as cited in Cohen (1964)]. Furthermore, there is evidence in the literature that ethylene accumulation in the vessel would affect growth variables negatively for most plants cultured in vitro (Arigita et al., 2003; Da Silva, 2013; Lai et al., 1998; Reis

et al., 2003). Therefore, a hypothesis for the necessity of a concentration of gaseous ethylene greater than 60 nL·L⁻¹ in the headspace to generate the biological response is unlikely to be true.

Explants did not alter ethylene production in the presence of ethephon in the culture medium ($P \leq 0.05$; Fig. 5A). There was evidence of small but measurable ethylene production by explants at the start of the experiment in the absence of ethephon ($P \leq 0.05$; Fig. 5B) that, as reported by others, may relate to a small temporary biosynthesis of ethylene resulting from wounding stress (De Paeppe and Van Der Straeten, 2005; Kende, 1993; Yang and Hoffman, 1984). In the presence of ethephon, explants of 'Showtime Diva' produced a little more ethylene than explants of 'Little Pinkie'. Although this may imply that 'Showtime Diva' has the capacity for an increase in its own ethylene production under the influence of ethephon, it was only 'Little Pinkie' that responded to ethephon as a shoot formation stimulant (Keshavarzi et al., 2014). This implies that the difference in sensitivity or response capacity of the tissue to ethephon drives differential cultivar responses (Firm, 1986). Together with results from previous experiments, ethephon is, therefore, unlikely to cause endogenous ethylene production in explants, but does trigger shoot formation in 'Little Pinkie' (Keshavarzi et al., 2014), perhaps through decomposition to ethylene and enhancement of the internal ethylene level possibly at a locally high concentration.

For the application in our study as well as other research using laser ethylene detector technology for ethylene measurement within unsealed in vitro culture vessels, for the first time we identified the sampling method as clearly superior over the continuous method. With the continuous flow method as reported by other researchers, the outflow was always constant, and the rate of ethylene release ended up at equilibrium, which was affected and limited by the air flow (Cristescu et al., 2008; Forni et al., 2012; Millenaar et al., 2009; van den Dungen et al., 2011). In contrast, however, using the sampling method, the rate of leakage could change depending on the gas pressure inside the vessel, as affected by any change in the rate of ethylene release from the medium: $CTm = (RMR - RL) \times t \times V$, where CTm is the concentration of ethylene at the time of measurement (measured in nanoliters per liter), RMR is the rate of release from the medium (measured in nanoliters per hour), RL is the rate of leakage from the vessel via the edges of the lids (measured in nanoliters per hour), t is the duration of accumulation in the vessel (measured in hours), and V is the volume of the container (measured in cubic meters). Flushing the air through the vessel resulted in continuous dilution of the atmosphere within the headspace that altered the gas pressure inside the vessel, giving rise to a different pressure from that normally found in the culture vessel when not continuously flushed with air, which therefore changes the rates of ethylene

Fig. 4. Number of secondary shoots developed per explant of gentian 'Little Pinkie'. For means separation, Fisher's unprotected least significant difference was applied to square root-transformed data; back-transformed values are presented. Columns not sharing a lowercase letter are significantly different and vertical lines represent $\pm$ SEM ($P \leq 0.05$, $n = 8$ vessels).

Fig. 5. Concentration of ethylene over time in the headspace of vessels containing either (A) agar medium amended with 10 mg·L⁻¹ ethephon with no explant (+E), or with explants of gentian ‘Showtime Diva’ (+E SD) or gentian ‘Little Pinkie’ (+E LP); or (B) agar medium with no ethephon containing no explant (-E), or with explants of ‘Showtime Diva’ (-E SD) or ‘Little Pinkie’ (-E LP). Averages were calculated using data from the repeated sampling method in unsealed culture vessels measured at only one time point every 2 d, recorded by ethylene detector (type ETD-300) at 5 L·h⁻¹ flow rate (n = 3 vessels each containing eight explants). A generalized linear model was applied to log-transformed data. Means comparison was made using the alternative least significant difference test, where the “Studentized” range statistic was used instead of Student’s *t* test at *P* ≤ 0.05. Actual mean values are presented here. Vertical lines represent ± SE.

production and concentration of ethylene in the headspace. In addition, for the first time we showed that in a unsealed system, the nonrepeated sampling method was preferred to the repeated sampling method, as it more closely simulates the actual conditions to which explants are exposed. With the non-repeated method, there was no significant difference in the concentration of ethylene in the headspace between measurement intervals (Fig. 1). This was presumably because, at the rate of ethylene production of 5.65 nL·h⁻¹ measured at the commencement of measurement time (4 h after adding ethephon to the medium) (Fig. 2), it would take only 2.64 h to produce 62.17 nL·L⁻¹ ethylene in the headspace, but the repeated samples were taken at 48-h intervals. Repeated sampling would be a less satisfactory method at lower rates of ethylene production or shorter sampling intervals. Also, although there was no significant difference between measured ethylene concentrations in the repeated and nonrepeated methods, the nonrepeated method was preferred because of the reduced risk of microbial contamination at each sampling time. The repeated method may still be useful in circumstances when plant material is limited. The optimized methodology used in our study would be beneficial to other future studies when measurement of ethylene at very low levels in unsealed vessels using ETD is required.

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