Propagation of Ornamental Plants
Vol. 00, № 0, 2000: 00-00

GASEOUS GROWTH REGULATORS: CAN THEY HELP IN SOLVING RECALCITRANCE PROBLEMS ENCOUNTERED WHEN ATTEMPTING TO CLONE PLANTS BY *IN VITRO* CULTURE?

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Abstract

For many species recalcitrance is still a problem when attempting to propagate plants by *in vitro* culture, especially when explants from adult individuals are used. In spite of extensive experimentation with variations in growth regulator concentrations and combinations, nutrient components and other factors commonly studied, efforts to propagate plants *in vitro* still often fail. This review looks at the possibilities of using gaseous growth regulator compounds in attempts to overcome such recalcitrance. The gaseous growth regulators most studied are ethylene and nitric oxide and the focus of this review will be on these. Less is known about carbon monoxide and hydrogen sulfide. These also have distinct morphogenetic effects and could perhaps sometimes be of use in solving recalcitrance problems.

Key words: carbon monoxide, ethylene, hydrogen sulfide, morphogenesis, nitric oxide, organogenesis, somatic embryogenesis

INTRODUCTION

Recalcitrance is a long-time problem that has frustrated efforts to propagate plants of many species by *in vitro* means, in particular when propagation of adult specimens is attempted. Recalcitrance is defined as the inability to obtain propagation by *in vitro* means by the use of traditional procedures such as manipulation of the mineral and organic contents of the nutrient medium, varying growth regulator concentrations and combinations and modifying physical factors such as pH, temperature and light regimes, venting of vessels etc. Recalcitrance problems are especially common for tree species as has been discussed in a couple of recent reviews (Bonga 2012, Bonga et al. 2010). Lately, a number of gaseous signaling molecules that have distinct functions in plant growth regulation, have attracted attention. The current review will consider whether some of these could potentially be of benefit in solving recalcitrance problems.

Plant development is controlled by various hormones and other plant growth regulators, most of these being in the auxin, cytokinin or gibberellin class. Besides these, it has long been recognized that gaseous ethylene also plays an important part. However, in the last few decades it has been found that a number of other naturally occurring gaseous compounds, nitric oxide being the most prominent of these, also have distinct effects on plant development. Plants produce an enormous number of gaseous products. Of the more than 100,000 chemicals that plants are known to produce at least 1700 are volatile (Dicke and Loreto 2010). A large number of these volatiles are involved in managing stress caused by physical damage or pathogens, or they act as attractants (Das et al. 2013). Some, however, distinctly affect growth and regeneration and could be of interest in situations where application of the traditional plant growth regulators has failed to overcome recalcitrance in *in vitro* propagation efforts. It is the objective of the current review to discuss a few of the volatiles that are known to affect growth and differentiation. The emphasis will be on the way these substances affect somatic embryogenesis (SE) and organogenesis (adventitious shoot and root formation, rooting of cuttings) because achieving proper functioning of these processes is required to overcome recalcitrance. To limit the scope of the review the discussion will primarily deal with recalcitrance in tree species, particularly in conifers.

ETHYLENE

The most investigated of the gaseous compounds is ethylene. It is a simple unsaturated hydrocarbon that is involved in regulation of many physiological processes

Received: August 1, 2013 *Accepted: August 30, 2013*

(Kumar et al. 1998, Ruduś et al. 2013). In some species, including a few woody ones, it can promote *in vitro* regeneration but in most it inhibits that capacity (Biddington 1992, Kumar et al. 1998, Dias et al. 2010). Ethylene is always an issue in propagation by *in vitro* means because it is produced by all cultures and accumulates in the air space of the culture vessels thus unavoidably exerting its growth regulating effects (Biddington 1992, Kumar et al. 1998, Arigita et al. 2003). Sometimes the effect is positive and often negative or ambivalent. The ethylene production rate is highest during intensive cell division, it decreases during cell expansion and increases again during maturation, senescence and tissue stress (Abeles et al. 1992). Accumulation of ethylene in the head space frequently inhibits organogenesis except in the case of rooting which it often promotes (Arigita et al. 2003). Generally it inhibits SE but in a few cases it has stimulated it, for example in the woody species *Coffea canephora* (Hatanaka et al. 1995). It is thought that ethylene may not inhibit the inductive phase of SE but inhibit its expression phase (Kumar et al. 1998). Ethylene enhanced induction of *Leucojum aestivum* SE and embryo development up to the globular stage while removal of ethylene with potassium permanganate resulted in improved subsequent elongation of the plantlets (Ptak et al. 2010). With regard to conifers it was found that non-embryogenic *Picea glauca* cell suspensions accumulated about three times more ethylene in the air space of the culture flasks than embryogenic cell suspensions (Kumar et al. 1990). Inhibition of growth in both cultures was due to inhibition by accumulation of ethylene in the airspace rather than by oxygen starvation. Similarly Wann et al. (1987) noted that non-embryogenic tissue of *Picea abies* produced ethylene at an about 8-times faster rate than embryogenic tissue. Similar results were obtained by Saly et al. (2002) with embryogenic and poorly embryogenic cell lines of *Larix □ leptoeuropaea* with the former producing less ethylene. Enrichment of the atmosphere with ethylene or addition of its precursors inhibited embryo formation while addition of ethylene inhibitors to the culture medium promoted it. El Meskaoui and Tremblay (1999) observed that maturation of *Picea mariana* SEs was better in sealed vessels than in vented ones and that sealed vessels prevented precocious germination. However, they concluded that the observed effects were not due to ethylene accumulation in the vessel but to reduced oxygen and increased carbon dioxide levels. Accumulation of ethylene and carbon dioxide in the airspace also promoted development and elongation of axillary shoots of *Thuja occidentalis* (Nour and Thorpe 1994). However, ethylene had a negative effect on *Picea glauca* SE maturation by inducing formation of intercellular air spaces in the shoot apical meristem (Kong and Yeung 1994). Adding an inhibitor of ethylene biosynthesis to these cultures reduced the number of embryos with abnormal meristems (Stasolla et al. 2002, Stasolla and Yeung 2003). In cultures of cotyledons of Pinus *radiata* accumulation of ethylene and carbon dioxide over a 10- to 15-day period in the vessel's airspace promoted adventitious bud formation (Kumar et al. 1987). Initiation of embryogenic cultures of *Pinus taeda* was promoted by the ethylene synthesis inhibitor silver nitrate in combination with Abscisic acid (ABA) (Pullman et al. 2003).

In a *Picea mariana* cell line that produced a large number of mature SEs ethylene biosynthesis was lower than in a line producing a low number of such embryos. Lowering the biosynthesis of ethylene with inhibitors increased embryo production in the line that produced a low number of mature embryos but had the reverse effect in the high producing line (El Meskaoui and Tremblay 2001). This indicated that ethylene synthesis in the low producing line was over optimal. Addition of silver nitrate to *Picea pungens* cultures inhibited embryogenesis initiation, suggesting that silver nitrate reduced endogenous ethylene to below optimal levels for embryo induction (Afele and Saxena 1995). In *Quercus ilex* high ethylene production was associated with secondary embryogenesis (Mauri and Manzanera 2011).

Two genes, *PsACS1 and PsACS2*, that encode the rate-limiting enzyme of ethylene synthesis 1-aminocyclopropane-1-carboxylate synthase (ACS) have been identified in embryogenic cultures of *Pinus sylvestris* (Lu et al. 2011). *PsACS1* was expressed during the proliferation and maturation stage of embryogenesis whereas *PsACS2* transcripts correlated with production of high numbers of cotyledonary stage embryos, the stage at which ethylene production is at its highest. The best embryo producing line generated lower levels of ethylene during proliferation and higher levels during maturation of the embryos than the lower embryo producing line.

The auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) is commonly used to initiate SE. It is thought that 2,4-D attains its effectiveness to do so in part by being an oxidative stressor and by increasing the cellular levels of ABA; in that process ethylene also becomes elevated (Fehér et al. 2003). Kvaalen (1994) found that in embryogenic cultures of *Picea abies* ethylene levels increased after removal of 2,4-D from the culture medium. This author suggested that ethylene may be involved in inducing SE and the early stages of embryo maturation of *P. abies*. It has been suggested (Chatfield and Raizada 2008) that the auxin and cytokinin normally supplied to induce shoot and root growth, may, if supplied in high concentrations, induce over-optimal concentrations of endogenous ethylene, thus negatively affecting regeneration. Pereira-Netto (2001) observed that 1-methylcyclopropene, an inhibitor that blocks ethylene receptors, stimulates multiple shoot formation in the fruit tree *Hancornia speciosa* and

that use of this inhibitor may be of use in propagating other recalcitrant species. In general, it is important to keep ethylene levels low during vegetative development to allow optimal expansion of the root and shoot (Ruduś et al. 2013). Trapping of ethylene or reducing its biosynthesis by inhibitors stimulated SE in cultures of *Hevea brasilensis* (Auboiron et al. 1990). On the other hand, adding an ethylene synthesis inhibitor to the culture medium of *Populus tremula* resulted in reduced induction and development of buds and roots while addition of ethylene synthesis stimulants promoted these (González et al. 1997). Similarly, ABA restricted ethylene production in *Populus* □ *canecens* and thus shoot growth (Arend et al. 2009). Contrary to this it has also been reported that shoot growth can be maintained by endogenous ABA because it partly suppresses ethylene synthesis (LeNoble et al. 2004). Whether ethylene acts as a promoter or inhibitor in these situations appears to depend on the levels of ethylene that are generated with low levels acting as promoter and high levels as inhibitor (Wilkinson and Davies 2010). ABA accumulation results in maintenance of root elongation by inhibiting ethylene formation (Spollen et al. 2000).

There are many reports indicating that ethylene stimulates rooting (Kurepin et al. 2011, Saini et al. 2013). In particular with conifers, ethylene application often promotes rooting (Ragonezi et al. 2010). Ethephon, an ethylene producing compound, in combination with auxin stimulated adventitious rooting in cuttings of *Pinus thunbergii* (Mori et al. 2011) and in those of other conifers (Ragonezi et al. 2010). For microcuttings *in vitro* ethylene is effective only for a short period immediately after making the cutting, i.e., during the root induction period (De Klerk 2002). Subsequently it becomes inhibitory, possibly because of inhibition of the establishment of polarity. Therefore, removal of ethylene from the headspace in the vessel at that stage is beneficial. Another factor to consider is the fact that auxin used for root induction raises ethylene production (De Klerk 2002). Sometimes inhibitors of ethylene biosynthesis will show the same effect as ethylene application, presumably because a high dose of these inhibitors creates stress and thus stress-induced overproduction of ethylene by the tissues (De Klerk 2002, Ragonezi 2010). The sequence of events in the initiation and elongation of adventitious rooting appears to be largely as follows (Kurepin et al. 2011). Auxin accumulates at the induction site which stimulates ethylene synthesis. This inhibits cytokinin formation, thus preventing cytokinin from becoming over-optimal during root initiation. Auxin synthesis diminishes after root initiation thus reducing ethylene formation which in turn reduces the inhibition of cytokinin formation. ABA inhibits ethylene production which promotes auxin transport into the roots which stimulates lateral root formation (Prasad et al. 2010). ABA accumulation results in maintenance of root elongation by inhibiting ethylene formation (Spollen et al. 2000).

NITRIC OXIDE

Nitric oxide (NO) is a component of industrial air pollution and is toxic in particular to the photosynthetic mechanism (Hill and Bennett 1970, Yamasaki 2005). However, it also acts as a plant hormone by being a gaseous signal transmitter that is involved in a wide variety of hormone-regulated processes (Yamasaki 2005, Simontacchi et al. 2013). NO bursts occur when plants are stressed. When provided by its donor sodium nitroprusside (SNP), it can sometimes mitigate damage caused by environmental stress, presumably by initiating proline accumulation (López-Carrión et al. 2008). At low concentrations it acts as a messenger that integrates stress mechanisms and defense against pathogens. At high concentrations it creates oxidative and nitrosative stress that causes premature aging and affects developmental programs (Durzan 2002, Durzan and Pedroso 2002). In contrast, exposure to NO can also reduce ethylene production and thus slow down aging (Leshem et al. 1998) and counteract senescence and oxidative stress by acting as an antioxidant (Hung and Kao 2003, Simontacchi 2013). Furthermore, it acts as an important signal in plant development (Durzan and Pedroso 2002, Arasimowicz and Floryszak-Wieczorek 2007). Wounding causes cellular NO bursts that cause DNA fragmentation (Garcès et al. 2001). This is an issue in *in vitro* propagation since wounding is unavoidable when excising explants or subculturing fragments of tissue. Hypergravity similarly results in a NO burst causing chloroplast DNA fragmentation and cell death in haploid megagametophyte cultures of *Taxus brevifolia*. This effect was also achieved by providing a NO donor to the culture medium and was counteracted by supplying a NO-synthase inhibitor (Pedroso and Durzan 2000, Pedroso et al. 2000). A discharge of *Araucaria* parthenospores into an *in vitro* culture medium is accompanied by a burst of NO synthesis (Durzan 2012). A NO burst is also associated with cleavage polyembryony in embryogenic cultures of *Picea abies* (Durzan 2002). In cultures of *Araucaria angustifolia* a correlation was observed between the reduced glutathione/glutathione disulfide (GSH/GSSH) ratio in the culture medium and NO emission. At low concentration GSH acted as a NO scavenger and stimulated formation of early embryos but inhibited polarization of these. At high concentration GSH stimulated NO formation and allowed formation of globular embryos and polarization (Nascimento Vieira et al. 2012). NO bursts initiate developmentally programmed cell death (PCD or apoptosis) which eliminates organs, tissues and cells that are no longer required for further development. This performs an important function in, among others, embryogenesis, organogenesis and apomixis (Durzan and Pedroso 2002,

Arasimowicz and Floryszak-Wieczorek 2007).

Polyamines, which are important in environmental stress control, rhizogenesis, SE and other developmental programs, induce rapid production of NO (Tun et al. 2006). Cytokinins have the same effect and the resulting NO appears to mimic cytokinin action by playing a role in cytokinin signal transduction (Tun et al. 2001). Spermidine and spermine supplied in the nutrient medium of embryogenic suspension cultures of *Araucaria angustifolia* inhibited formation of NO but promoted normal development during the early stages of embryogenesis. Putrescine increased endogenous NO production and its accumulation was higher in embryonic cells than in the suspensor suggesting that NO release may play a function in the maintenance of polarity (Silveira et al. 2006). Spermidine and spermine play a role in SE development and maturation of the rainforest tree *Ocotea catherinensis* by altering its endogenous NO metabolism (Santa-Catarina et. al. 2007). These authors suggested that endogenous NO could play a role in the acquisition of embryogenic competence in plant cells.

NO donors reduce ethylene production in cell cultures (Lindermayer et al. 2006) and can thus perhaps sometimes be useful where ethylene is a problem in propagation through SE or organogenesis. Another aspect of NO application that may affect *in vitro* experimentation is the fact that it stimulates the formation of ABA (Arasimowicz and Floryszak-Wieczorek 2007) and thus may act as a maturation and senescence regulating factor (Leshem et al. 1998). This perhaps may, for example, promote proper maturation of SEs. In apple seed NO treatment stimulated germination and perhaps increased embryo sensitivity to endogenous ethylene, thus stimulating hypocotyl and cotyledon growth. Furthermore, the NO application stimulated reactive oxygen species formation which probably activated radicle growth (Gniazdowska et al. 2010). It was also observed that supplying the NO donor sodium nitroprusside (SNP) to the *in vitro* culture medium of *Malus hupehensis* promoted shoot multiplication and root formation (Han et al. 2009). Other NO effects that have occurred *in vitro* are activation of cell division and formation of alfalfa embryogenic cell clusters that expressed the *Ms*SERK1 gene, a gene implicated in somatic and zygotic embryogenesis. NO activated the cell cycle but was not involved in its progression (Őtvős et al. 2005). SNP reduced browning and promoted callus induction and shoot regeneration in cultures of *Dioscorea opposita* (Xu et al. 2009) and stimulated adventitious shoot formation in *Vanilla planifolia* (Tan et al. 2013). In callus cultures of salt intolerant *Populus popularis* and salt tolerant *P. euphratica* exposure to salt stress induced higher levels of endogenous NO in cultures of the salt tolerant species than in those of the salt intolerant one. This indicated that the increased NO level in the *P. euphratica* cells enabled them to deal more effectively with reactive oxygen species than the cells of *P. popularis* (Sun et al. 2010). A five-day treatment with a NO donor stimulated shoot formation in cultures of the tree legume *Albizzia lebbeck* (Kalra and Babbar 2012).

NO plays a major role in adventitious root formation and growth of several species (Simontacchi 2013) by acting downstream of auxin in the signaling pathway that leads to root development, a process that involves cytosolic Ca^{2+} through a calmodulin-dependent mechanism (Lanteri et al. 2008, Chen and Kao 2012). It also appears to promote adventitious rooting by acting downstream from indole-3-acatic acid (IAA) by activating the important cellular messenger cyclic guanosine monophosphate (cGMP) and mitogen-activated protein kinase (MAPK) (Pagnussat et al. 2003, 2004). Treatment with a NO donor promoted rooting in both relatively easy to root juvenile cuttings and difficult to root mature cuttings of *Eucalyptus grandis*. Endogenous NO production was about twice as high in the non-treated juvenile cuttings than in the mature ones (Abu-Abied et al. 2012).

MISCELLANEOUS GASEOUS COMPOUNDS

Various other gaseous compounds have morphogenic effects although these have been less well researched than those discussed above. A few examples are the following.

Exogenous carbon monoxide (CO), a toxic gaseous compound, appears to have morphogenic effects in plants. It promoted lateral root formation by increasing the level of endogenous NO (Cao et al. 2007, Guo et al. 2008) and initiated root hair development by acting synergistically with auxin, ethylene and NO (Guo et al. 2009). Similarly Xuan et al. (2008) found that CO is involved in auxin-induced adventitious rooting of cucumber hypocotyls by acting downstream of auxin in a signaling cascade. They also cite several references for other species where CO stimulated adventitious rooting. Hydrogen sulfide (H_2S) is another gaseous endogenous signaling transmitter besides CO and NO (Wang 2002). It stimulates root formation by acting upstream of endogenous IAA and NO in signal transduction (Zhang et al. 2009). Some jasmonates, the ones used for communication between plants, are volatile. Jasmonate counteracts Gibberellic acid (GA) and thus has a function in diverting resources from growth to defense against stress conditions (Lyons et al. 2013). It also has a function in lateral root formation by interacting with auxin (Saini et al. 2013).

CONCLUSION

All gaseous compounds discussed above have a function in morphogenesis in particular in root forma-

tion. In their control of plant development they all interact with each other in complex ways and whether each acts as a stimulator or inhibitor depends on their status within these interactions. It is of interest to note that all have a function somewhere in the signaling cascade involving IAA and thus often affect morphogenesis by the way they affect the action of IAA. How useful exposure to these volatiles can be in solving recalcitrance problems is difficult to ascertain. It is conceivable that if the recalcitrance problem is associated with a malfunctioning somewhere in the IAA signaling cascade that application of one of the gaseous compounds could mitigate the problem. These compounds may be less useful if the problem is associated with physiological processes largely outside the IAA signaling cascade.

As was pointed out above, NO application sometimes has a positive effect on various stages of SE and this deserves further exploration. NO is involved in programmed cell death control. This is an important aspect of the proper functioning of SE formation and in organogenesis and exogenous application of NO could perhaps help improve these processes. Furthermore, NO often has a stimulating effect on root formation. Proper root formation is often a stumbling block in propagation by SE or organogenesis and application of NO could, therefore, in some cases, be of assistance.

It is more difficult to discern if application of ethylene or inhibiting its biosynthesis could be effective in promoting SE or organogenesis. The literature dealing with that subject is often ambivalent or contradictory. The few reports that have been published on the subject indicate that how SE or organogenesis are affected by ethylene *in vitro* is determined in a sensitive fashion by endogenous ethylene levels. Optimal endogenous levels vary with the stage of development and it is essential that these are strictly maintained for each developmental phase in propagation experiments. At each stage of development this requires lowering of ethylene levels in the airspace of the culture vessel by ventilation or use of ethylene adsorbents when the endogenous level is too high, or by supplementing the culture medium with ethylene generating compounds when it is too low. The literature is far clearer with regard to rooting. In many cases ethylene application has stimulated rooting, primarily by interacting with IAA. Proper root development is often a problem in SE and ethylene application may be helpful in some cases where rooting is aberrant. CO and hydrogen sulfide (H_2S) similarly can perhaps sometimes be effective in overcoming recalcitrance by stimulating rooting. For these gases similarly a strict control of their endogenous concentrations to a level appropriate for each stage of development during the regeneration process is essential.

Aberrant genomic expression *in vitro* is associated with stress (Joyce et al. 2003), which leads to such abnormalities as, for example, hyperhydricity (Saher et al 2005). Abnormalities thus created presumably are a main cause of recalcitrance. We can reasonably assume that abnormalities associated with stress are often due to our inability to provide precisely the same conditions during the *in vitro* process that prevail during the various stages of development *in situ*, the latter being highly fine-tuned and constantly changing with each phase of development. Therefore, careful optimization of tissue culture protocols still remains a necessity when trying to solve recalcitrance problems and this includes paying attention to the involvement of gaseous compounds. Further research with gaseous compounds is required to establish how useful they can be in routine clonal propagation procedures.

Acknowledgements: I wish to thank Dr. Krystyna Klimaszewska and Dr. Patrick von Aderkas for their critical review of the manuscript and helpful suggestions.

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