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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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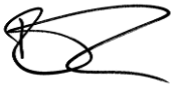
Signature 

Date 11/11/12

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GROWER SUMMARY

- The stock plant environment is a contributory factor in successful adventitious root formation.
- There may be optimal soil moisture ranges for stock plants for subsequent rooting that reflect changes to plant physiology.
- Abscisic acid and ethylene are plant stress hormones involved in adventitious root formation.

Background and expected deliverables

There is a great variability between hardy nursery stock subjects in the ability of shoot tissue to produce adventitious roots (AR). Adventitious roots are a subtype of roots that emerge from cuttings and their production is integral to the propagation of countless numbers of elite varieties worldwide, which are of use in horticulture, forestry and timber production, food production and for conservation. It is a complex trait in plant development and is dependent on the interaction between many environmental and internal conditions. However, at the core of the process is the plant growth regulator auxin, long used as an effective synthetic treatment as one of its many analogues in cutting propagation. The plant growth regulator ethylene has also been demonstrated to have a generally promotive role in the rooting process and there may be roles for other environmentally regulated 'stress' hormones such as abscisic acid (ABA).

Most propagation professionals will be unsurprised by the variability in rooting in some varieties on a yearly basis. Although this variation is most often left unexplained, it is most-likely produced by the effect of climatic variations in season from year to year. Stock plants used for cuttings are not always subject to as rigorous a management regime as the subsequent plants that are produced from them, which is somewhat surprising given that the tissue used to produce the new plants has spent most of its time attached to the parent plant. Recent discoveries showing how genes are controlled are giving us more information as to how environmental changes can produce stable alterations in the way genes function within plants, which may even be inherited by sexually-propagated offspring, but most certainly through asexually-propagated cuttings.

- This project is therefore investigating how soil moisture of stock plants can affect subsequent rooting of cuttings and whether this effect is produced by changes in hormone signals.
- It is hoped that the investigation will develop practical advice that will allow nurserymen to produce improvements in rooting from all stock, but especially problem rooting species or varieties.

Summary of the project and main conclusions

First year studies identified a role for ethylene in adventitious rooting from woody species and also trialed the use of ACC (ethylene precursor) as a rooting treatment, however, this proved to be ineffective solution in terms of feasibility and cost.

The study has progressed to focus on the environmental regulation for adventitious rooting in stock plants as outlined in the introduction. Stages in the study are:

1. Does soil moisture content in stock plants influence subsequent rooting?
2. Does this response involve variation in endogenous ethylene and ABA synthesis and response?
3. How stable is any effect in stock plants over time?
4. An online grower survey into stock plant management with respect to irrigation.
5. Field study in local nurseries.
6. Trials for a wide-range of species.

Current results indicate that there may be ranges of soil moisture that promote rooting and that these may be different for different species.

Hypothesis

The hypothesis is that stock plants subject to drought or over-irrigation will increase ABA and ethylene hormone levels to concentrations where they inhibit root formation. The hypothesis is not based on a direct ratio of the two hormones, but on thresholds for each hormone in different moisture ranges. Some species may be less sensitive either to ethylene in waterlogged conditions or ABA in droughted conditions, rendering differences in their potential range of rooting.

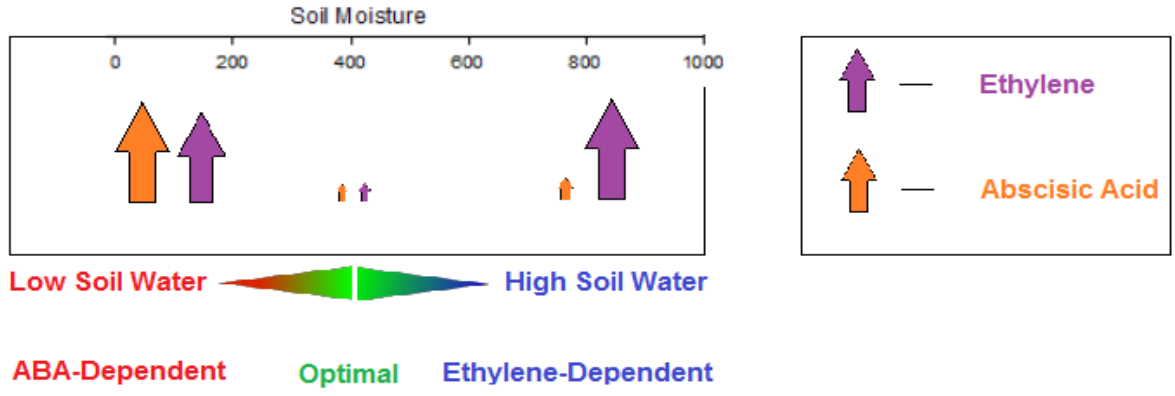

Fig. 1. Hypothetical variation in ABA and ethylene concentrations over the soil moisture range in soil-based substrate, based on literature and current data. Increases in the concentrations beyond the 'permissible' ranges may produce changes that inhibit root induction. Note that there are two distinct 'stress' zones which are primarily dictated by individual hormones.

Year 2: Main Section

1: Water Deficits in Stock Plants

Our current research has identified potential optimal ranges for soil moisture in stock plants for rooting. Experiments investigated the effect of reducing soil moisture in stock plants on cutting rooting. There were two treatments: a well-watered (100% of evapotranspiration; ET) and a deficit-irrigated (50% ET) treatment. Cuttings from deficit-irrigated plants exhibited a statistically significant 26% reduction in rooting after 10 days compared to the well-watered controls. Results from experiments in two *Populus* species both show an optimal response to a defined range of soil moisture (Fig.2.). This may mean that there are specific soil moisture ranges for stock plant for which rooting is promoted or suppressed. In addition, cuttings removed from deficit treated plants showed increased ethylene production at 24 hours after excision to levels that may be inhibitory. Although moderate levels may be stimulatory, higher or lower levels may inhibit root induction.

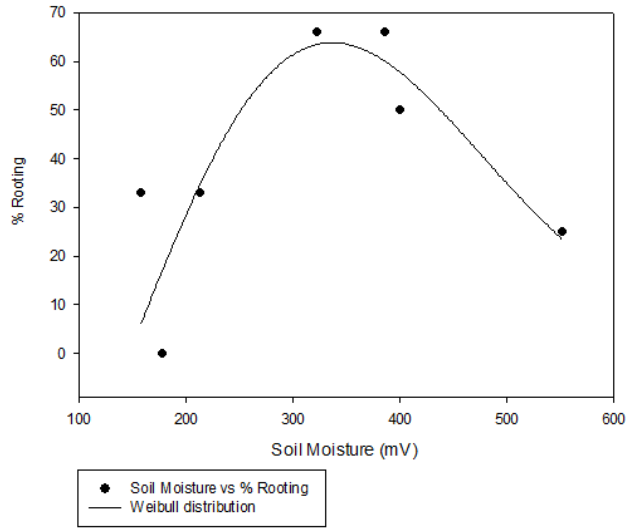

Fig. 2. An optimal soil moisture range for stock plant irrigation for adventitious rooting?

2: A Role for ABA in Adventitious Rooting?

ABA and ethylene are known to be antagonistic in a number of plant processes. ABA has been dubbed the 'water stress hormone' owing to its accumulation and role in plant water relations under drought conditions. Therefore, to initially examine if there is any role for ABA in adventitious rooting we compared rooting by tomato varieties overproducing ABA (sp12 and sp5) and the wild-type, ‘Ailsa Craig’. Results indicate that percentage root emergence after 10 days was negatively associated with increased ABA production and, in addition, overproducing mutants had significantly lower ethylene concentrations during the first 24

hours after cutting excision. This provides evidence for a possible role for ABA and an interaction with ethylene in the promotion of AR and therefore gives further justification for studies into how rooting is affected by soil moisture variation.

3: Current Experiments

To clarify if there is any role for stock plant soil moisture in priming for AR and whether variation exists between species, I am currently conducting a larger scale experiment using four *Populus* varieties. The methodology is split into three experimental stages (Fig.3 below) (1) the acclimation period wherein all plants get the same irrigation and are kept within the same soil moisture range; (2) the treatment period wherein there are three different soil moisture treatments corresponding to a low, medium and high soil moisture content and (3) the post-excision or cuttings period, wherein the physiological and rooting response of the cuttings are measured under constant conditions. In all stages various physiological and biochemical data is taken.

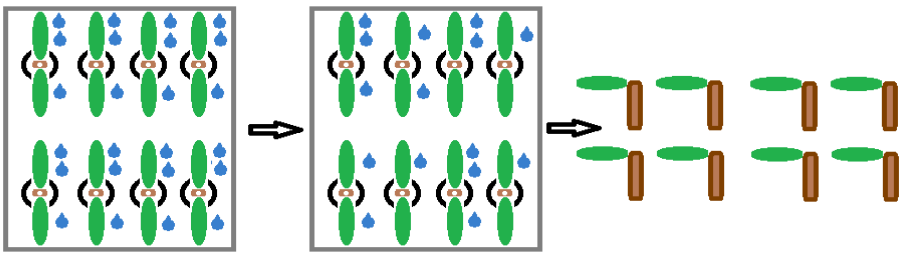

Fig.3. Experimental work-flow for the larger scale soil moisture priming experiment showing the progression from acclimation stage with constant water, to treatment stage with 3 moisture regimes and finally to the cuttings.

Preliminary results indicate that there are promotive ranges for rooting but that effects on rooting may be species-specific, possibly relating to the degree of innate drought/flooding tolerance of each species and their production of ethylene and ABA.

Financial benefits

Development of new stock plant management recommendations provides an easy method of increasing the percentage of rooting (reduction in losses) for a range of recalcitrant and non-recalcitrant species and could allow greater market exposure for species previously considered as being unprofitable due to low rooting response. The adoption of such processes could help to more effectively target water use and mitigate the use of synthetic chemical rooting agents, many of which add significant costs to business and the environment and will be restricted under new EU directives by 2020. In addition, such

knowledge can be incorporated into training. Water use is a significant problem for the horticultural industry, both in financial and environmental terms and over-irrigation remains a problem.

Action points for growers

1. Over- or under-irrigation of stock plants may lead to undesirable changes in plant tissues as well as variation in ABA and ethylene levels outside of the conducive range for ARF, although the level of drought tolerance of the species should be considered when making judgements about irrigation.
2. Growers can improve their practice by incorporating simple record keeping of the environmental variables to which stock plants are subjected on a seasonal (or even monthly) basis as a means to explain losses and in helping to optimise treatment.

SCIENCE SECTION

Introduction

There is a great variability between hardy nursery stock subjects in the ability of shoot tissue to produce adventitious roots (AR). Most propagation professionals will be unsurprised by the variability in rooting in some varieties on a yearly basis. Although this variation is often left unexplained, it is most-likely produced by the effect of climatic variations in season from year to year. However, at the core of the process is the plant growth regulator auxin, long used as an effective synthetic treatment as one of its many analogues in cutting propagation. The plant growth regulator ethylene has also been demonstrated to have a generally promotive role in the rooting process and there may be roles for other environmentally regulated 'stress' hormones such as abscisic acid (ABA). Recent discoveries showing how genes are controlled are giving us more information as to how environmental changes can produce stable alterations in the way genes function within plants, which may even be inherited by sexually-propagated offspring, but most certainly through asexually-propagated cuttings.

The project was initially focussed on the role of the plant hormone ethylene as a promoter of adventitious rooting. Studies from the first year of the project identified a role for ethylene in adventitious rooting and also similar roles in both recalcitrant and rooted species (refer to first year report) and identified that ethylene is most effective at promoting rooting in cuttings around 24 hours after excision from the plant.

However, as much work has already been conducted into the role of ethylene in adventitious root formation (ARF) (Ivanchenko, Muday, & Dubrovsky, 2008; Konieczny *et al*., 2009; Negi, 2010) and greater complexity exists (Ramírez-Carvajal *et al*., 2009). Therefore, the project shifted in focus from the cuttings themselves to the conditions produced by exposure to environmental factors in stock plants, of which hormonal interactions play a major role.

Recently research has highlighted the importance of epigenetic control by the environment to which an organism is exposed. Epigenetics is the control of gene expression where genes can be modified and switched ‘on’ and ‘off’ without altering the DNA sequence. There is evidence that the environment influences plant responses, for example with relation to vernalization and flowering (Kim, Doyle, Sung, & Amasino, 2009) salinity (Lira-Medeiros et al., 2010) and more generally (Raj et al., 2011).

Several studies in plants have confirmed a role for these accumulated DNA modifications (Baubec et al., 2010; Chinnusamy & Zhu, 2009; Lisch, 2009) and even trans-generational inherited acquired states (Boyko & Kovalchuk, 2011 and Saze, Mittelsten Scheid & Paszkowski, 2003). The mechanisms by which such modifications are produced are only just starting to be investigated and therefore information is sparse. There are, however, some candidates likely to be involved in producing these changes under environmental stress. Plants produce a number of quickly induced signals in response to changes in environmental conditions and two important hormonal signals are ethylene and abscisic acid (ABA). Both are involved in altering the morphology and physiology of plants in short-term acclimation to the environment in addition to potential roles in regulating long-term change via priming cells for future response.

The more immediate roles of ethylene in the formation of adventitious roots have become clarified in recent years (Konieczny et al., 2009; Negi, Sukumar, Liu, Cohen, & Muday, 2010), especially its role in adjusting auxin transport, perception and signalling, which is crucially important for the induction of cell division from meristematic tissue (cambium) at the rooting site (Dello Ioio et al., 2007; Ruzicka et al., 2009; Skylar, Sung, Hong, Chory, & Wu, 2011). Ethylene may have the capability to induce meristematic cell division in some species under certain conditions (Love *et al.,* 2009). Since the synthesis and activity of ethylene may vary with certain environmental variables, such as soil moisture in the case of flooding (Peng et al., 2005), it is an important consideration in ARF.

ABA is rapidly synthesised under drought stress conditions and also catabolised rapidly when such conditions abate. It is involved in the regulation of a number of physiological processes that allow for effective stress acclimation, such as stomatal closure. Some of the underlying molecular events in the early rooting process may be affected by ABA. ABA concentration may control the expression of a number of the miRNA molecules (Liu & Chen, 2009) that target transcription factors involved in auxin signalling identified as regulating ARF (Gutierrez et al., 2009). ABA may also regulate ethylene signalling (Wilkinson & Davies, 2010; Wilmowicz, Kesy, & Kopcewicz, 2008) to fine-tune physiological responses to environmental stresses.

This study therefore seeks to investigate whether variation in soil moisture produced by inadequate irrigation management could contribute to losses through reductions in cutting rooting because of altered ABA and ethylene signalling and whether these conditions lead to a change in the response of excised tissue. Very little work has ever

been conducted in this area of pre-excision environmental conditioning and so this study acts as a bridge to further focus stock plant management for cutting propagation.

In addition, some work was conducted on the influence that variation in red:far red ratio in stock plants may have on eventual rooting of cuttings. Some of the preliminary physiological data is displayed at the end of both the methods and results sections. Another trial experiment using red:far red ratio manipulation will be conducted using selective sheeting as a further medium-term pre-excision treatment of stock plants. A low red:far red ratio stimulates auxin and ethylene responses that may be relevant to adventitious rooting (Morelli *et al.* 2000; Stamm & Kumar, 2010 and Tao *et al.* 2008). Differences may also create epigenetic changes that may alter later plant responses (Klose et al., 2012).

Methods

Initial Study

Thirty plants per species (80-100cm b/r 1+1 *Populus nigra* and *Populus tremula*) were potted up into sterilized John Innes No.3 media (LBS Horticulture, Colne, Lancashire, UK) contained in black 5L pots and left to acclimate in a protected environment for seven days at 25^0C with a 12:12 (light:dark) cycle with a fully watered (defined as 1.0 ETp; Potential Evapotranspiration) regime after first leaf-set. Irrigation was performed manually, introducing two treatments on the initially well-watered stock plants. All plants were weighed daily and the evapotranspiration estimated after 24 hours using water loss measurements (Sharp et al. 2009). Treatments were 1.0 ETp and 0.5 ETp per day. Rootzone moisture was measured using a standard HH2 soil moisture meter with theta-probe (Delta-T devices, Cambridge, UK) every two days during the treatment and stomatal conductance readings taken three hours after watering on the same day using a Model AP4 steady-state porometer (Delta-T devices, Cambridge, UK).

Three dual-nodal lateral cuttings per stock plant were removed between 08:00 and 09:30 after 14 days of treatment. Leaves were surface sterilized for five seconds with 5% v/v domestos solution (Unilever, Surrey, UK), washed in de-ionised water, placed in Braam paper plugs (Fargro, Littlehampton, UK) filled with a peat:vermiculite:perlite mix (3:1:1) and arranged in a randomised complete block design (RCBD) with perimeter guard samples to account for edge-effects. Five second mist bursts were regulated by an Evaposensor (LBS, Colne, Lancashire) set at medium sensitivity. Five cuttings per treatment×species were frozen after sterilisation in liquid nitrogen for ABA quantification. An additional five cuttings per treatment×species were basally sealed in vials containing

the Braam media for ethylene gas extraction at over time-course of rooting and arranged in an analogous layout to unenclosed cuttings. Cuttings were enclosed in a 70ml test tube for 1hr in order to measure variations in ethylene evolution over time-course of rooting at 0h, 6h and 24h post-excision. A 4ml sample of headspace ethylene from the cuttings was extracted by a luer-lock gas-tight syringe equipped with a removable 23ga SGE Luer Lock needle (Sigma Aldrich, UK) and stored in a pre-vacuumed 3.7ml Exetainer vial (Labco, Lampeter, UK) (Glatzel and Well 2008).

The ethylene samples were quantified by injecting 1ml into the column of an Agilent Gas Chromatograph-Mass Spectrometer (GC-MS) with coupled flame-ionisation detector (FID). Peak area integration was used to estimate ethylene concentration as nL $g^{-1}FWh^{-1}$.

Extended Study Including Four Species (Current Work: Ongoing)

Twenty cuttings per species (*Populus adenopoda (from previous stock propagated from Kew Gardens: 1981-8644), P. mariesiana (from previous stock propagated from Kew Gardens: 1973-21312), P. nigra ssp. betulifolia (from previous stock propagated from W. Crowder and Sons, Horncastle)* and *P. tristis (from previous stock propagated from Kew Gardens: 1969-12379))* were propagated in Braam paper plugs filled with a peat:vermiculite:perlite mix (3:1:1) and left for 7-18 days until rooted. Once roots were protruding from the plugs cuttings were transplanted into 5L pots filled to 4L with sterilized John Innes No.3 media and fertilised by topdressing with Osmacote Exact Mini pellets (LBS, Colne, Lancashire, UK) at a dose of $2gL^{-1}$ and kept in semi-controlled greenhouse conditions with a mean ambient temperature of $25^0C \pm 4^0C$, a mean PAR of 400-500µmol m^{-2} s^{-1}, a R:FR of 1:3 over a 16:8 (light:dark) cycle. Individuals were watered every two days to full ETp as indicated by gravimetric measurements using an Adam balance. After at least two weeks and four weeks prior to treatments, the apical meristem of each plant was removed to induce branching. Over four days preceding treatments the following morphological and physiological variables were recorded every two days from leaves with a plastochron index of approximately +5, chosen to reflect a mean developmental stage for basal portions of cuttings:

- Stomatal conductance (g_s) (Delta Devices AP4 diffusion porometer)
- Net photosynthesis (*A*) (LICOR LI-6400XT Photosynthesis System equipped with 6400-02B Red/Blue LED Light Source; LICOR Biosciences, Bad-Homberg, Germany). PAR was set to 1000µmol m^{-2} s^{-1}. The flow-rate of air through the sample chamber was set at 350µmol s^{-1}, the leaf temperature was 25^oC and the CO_2 concentration of the sample chamber was adjusted to $390\mu molL^{-1}$ using a

CO_2 injector (6400-01; LI-COR).

- Whole-plant transpiration estimated using water use and leaf area
- Soil moisture (Delta-T HH2 soil moisture meter with ML2X theta-probe)
- Growth rate calculated from main stem height (image calculation in imageJ)
- Plastochron index
- Chlorophyll fluorescence (F_m/F_v) (Pocket Pea; Hansatech Instruments, King's Lynn, UK) (at same time as stomatal conductance)
- Lengths of the two newest emergent leaves from the longest shoot.

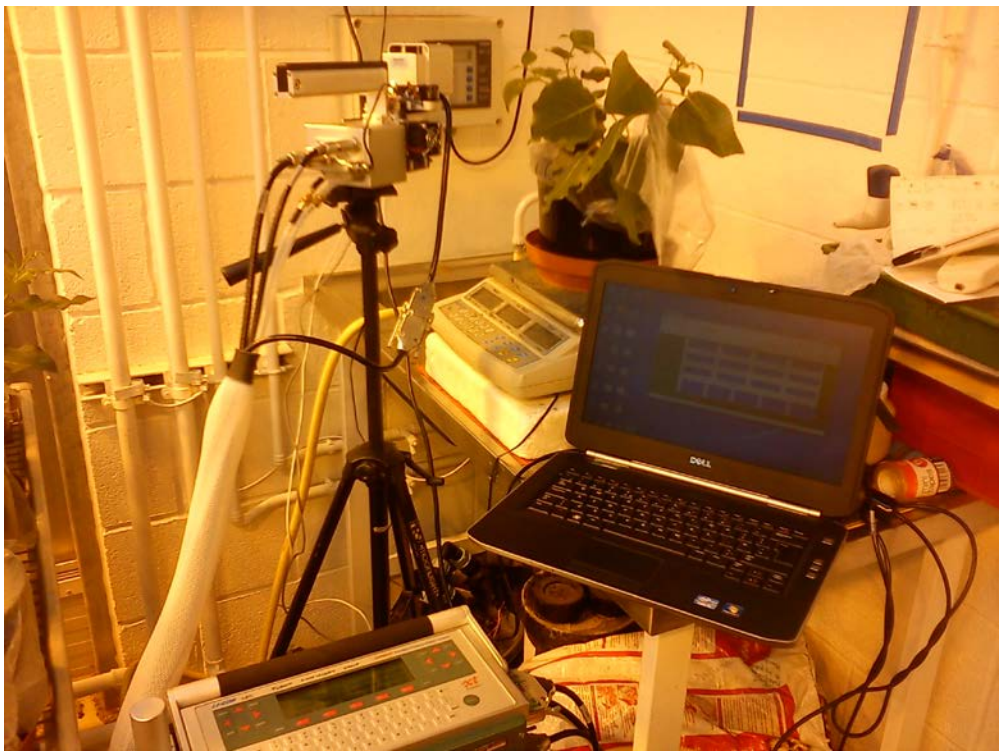

Fig.1. The LICOR LI-6400XT Photosynthesis System used to record various physiological variables from plants in the study.

All measurements were paired recordings and conducted between 11:30 and 14:30. For each measurement set PAR readings were taken so that light could be included in the data analysis. The plastochron index was calculated by measuring leaf expansion over a defined time period and using the equation below with reference leaf lengths defined for each species from the period of maximal expansion rate:

$$\mathrm{PI} = n + \frac{\ln L_n(t) - \ln \lambda}{\ln L_n(t) - \ln L_{n+1}(t)}.$$

Where n is the serial number of the leaf (measured acropetally), λ is the reference leaf length, L_n is the length of the leaf longer or equivalent to the reference value and L_{n+1} is

the length of the subsequent leaf smaller than reference value. With the plastochron calculated, the leaf plastochron index (LPI) could be calculated using:

$$\mathrm{LPI}_i = \mathrm{PI} - i$$

Where LPI, Leaf plastochron index; PI, Plastochron for the n^{th} leaf; I, Serial number of the leaf numbered basipetally.

Fig.2. Progression in leaf area with age in *Populus mariesana.* Predictable leaf expansion rates allow for the scaling of the relative age of the tissue using the plastochron index.

Leaf water potential was measured for each plant from the same leaf using a Thermocouple Psychrometer; model C-52 Westcor sample chamber attached to a HR-33 Dewpoint Microvoltimeter (Westcor Biomedical Systems, Utah, USA) on the day before the initiation of treatments. Instantaneous water use efficiency (WUEi = A/E) was calculated by dividing photosynthetic rate by transpiration and net carbon assimilation was estimated using the equation CO_{2in}-CO_{2out}. In addition, free sucrose (total free glucose-glucose on addition of invertase) was quantified from both leaf, stem and petiole tissue using sucrose assay kits from (Sigma Aldrich UK).

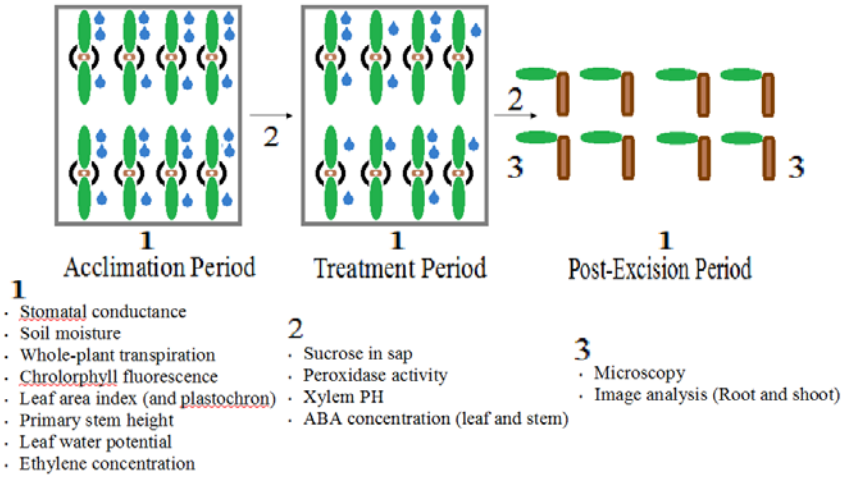

Fig.3. Workflow for the experiment, showing the three stages of Acclimation (10 days), Treatment (10 days) and Post-Excision (10 days) and the measurements taken within each time-point. Measurements (1) were taken every three days during the respective phases, (2) were taken during transitions between stages when plant material could be gathered and (3) at the end of the experiment.

Tissue samples were placed in 1.2ml eppendorf tubes immediately after excision from the plant, flash-frozen in liquid nitrogen and stored at -20^{0}C until analysis. Approximately 200mg samples of leaf tissue and petioles were lyophilised (freeze-dried) at -80^{0}C for 24 hours in a complete vacuum. Dried samples were then ground and 1.1ml of 80% aqueous ethanol was added. Samples were placed in a water bath at 80^{0}C for 1 hour for extraction and centrifuged at 5000g to remove cellular debris. Supernatants were extracted and assayed using the invertase enzymatic method with spectrophotometric determination at 340nm using a commercial kit (SCA20-1KT Sigma Aldrich, UK). Analysis was performed in 1mL volumes at 25^{0}C in quartz cuvettes.

One plant per treatment population was used for ethylene quantification at three day intervals. To measure ethylene evolution from intact plants, one leaf of each sampling plant was enclosed in a sealed plastic 750ml bag for one hour one day between 10:30 and 11:30 in leaf and petiole of similar physiological age based on plastochron measurements. These bags were further sealed at the edges with petroleum jelly and 1 ml of air from the hole was extracted using an SGE 1ml gas-tight syringe. As a control, 5 bags were incubated for 1 hour in a 42ml glass vial and emitted ethylene measured using the GC-FID method outlined previously. Results indicated that under 0.005nL h^{-1} ethylene was emitted by the bags under standard conditions (standard deviation 0.003) and this was included when calculating plant-derived ethylene.

Treatment Phase

A 12-factorial (4×3 factor) experimental setup was designed that allocated 3 ranges (16 plants per species×soil moisture) of soil moisture that broadly spanned 150-349 mV, 350-549mV and 550-749mV) produced by watering at 0.33ETp, 0.66ETp and 1.0ETp respectively every 3 days based on individual plant gravimetric measurements using an Adam balance and the rate of decrease in soil moisture using an equation formulated by the author (Appendix I).

The soil surface of each pot was covered with polyethylene film at the stem base to prevent surface evaporation and collection trays were used for every plant to prevent loss of water to the external environment and therefore maintain intended soil water status. Three replicates; four plants in each replicate were conducted to minimise sampling errors and to allow for more effective statistical manipulation. During this treatment period, the same physiological and morphological variables were recorded every two days as previously described in the preceding section.

At the end of the treatment phase all plants were re-watered to a fully watered (1ETp) state for two days before excision of cuttings.

Cutting Phase

When the plants were within the intended soil moisture range for three consecutive days, cuttings were taken, as described previously, and placed in shallow trays covered with transparent film in order to optimise humidity (measured at ~75% RH). Reference plugs were included in each tray to monitor and correct changes in media moisture. Physiological measurements (as previously described in the **Treatment Phase** section) were taken for cuttings at 0, 24 and 96 hours post-excision and after 10 days. In addition, ethylene measurements were taken at these time points using gas chromatography with flame-ionisation detection (GC-FID) quantification using methods already described in the first section of the **Methods; Extended Study**.

Upon excision, the leaf water potential of cuttings was measured and tissue samples were analysed for sucrose, as described previously in **Methods; Extended Study**. Samples were sectioned into leaf tissue, stem tissue and roots (if present) and stored in liquid nitrogen before storage at -20^{0}C until assayed for ABA and sucrose/glucose as outlined in **Methods; Extended Study**. The ABA concentrations were determined using a radio-immuno assay method (Quarrie et al,1988). Stem segments of ~3cm were quickly cleaned in 5% Tween solution, washed in de-ionised water and then freeze-dried

at -40^{0}C for 28 hours, followed by aqueous extraction in 1ml eppendorf tubes with centrifugation at 15,000RPM for 30 minutes. The supernatant was aliquoted into separate tubes and stored at -20^{0}C until a radioimmuno assay using the monoclonal antibody AFRC MAC 252 (kindly provided by Dr S Quarrie).

During this cuttings period, the physiological and morphological variables listed in both previous sections were again recorded every three days. After 10 days, rooting, rooting area, primary root lengths and number of laterals were analysed from images produced with a standard 5MP digital camera using the Smartroot plugin (Universite Catholiqué de Louvain) for imageJ.

Fig.4. Taking leaf water potential measurements from cuttings before sectioning sing the Westcor H-33 and C-52 chambers.

Histological investigation of horizontal (cross) sections of sample cuttings were conducted using both iodine staining for amyloplasts (starch granules) and safranin o staining (ultrastructural examination) with light microscopy. Cutting bases of 5mm were fixed for 24 hours in a 3:1 ethanol: acetic acid solution before sectioning with a Leica microtome. Slides were stained for 2 hours in 0.6% ethanolic safranin o solution, washed with progressively decreasing concentrations of ethanol and finally, examined on a Watson light microscope at 40x magnification.

Univariate analysis of data will compare between treatments to correlate physiological and biochemical indicators of water stress with final rooting response.

Investigating the role of ABA in root induction

The experimental design was a random arrangement of 20 *Solanum lycopersicum* 'Ailsa Craig', sp12 and sp5 (both ABA overproducers) respectively, which were sown into vermiculite medium supplemented with half strength Hoagland's solution. The sp5 seeds had been pre-treated with the herbicide norflurazon to assist germination. Once the first true leaves were emergent, plants were transferred into 1L pots containing sterilised John Innes No.2 media. Plants were grown until they reached the 5 leaf stage and cuttings were taken from the pre-floral node for standardisation as has been previously outlined and arranged in a random arrangement in braam propagation plugs containing a peat:vermiculite:perlite mix (3:1:1) and left for 10 days until sampling. Data on root number, length and percentage were recorded. Statistical differences between genotypes were assessed via a student's t-test for average root number and length.

Investigating Red:Far red ratio

Three mixed wavelength variable intensity arrays (Glolighting (Agrilamp), Derby UK) were used to create two environments with different red:far red ratios: low (0.39) and high (4.1) with constant intensity. Ten stock plants respectively of (80-100cm b/r 1+1 *Populus nigra, P. tremula* and *Acer campestre*) were potted up into sterilized John Innes No.3 media contained in 5L pots and kept to acclimate for seven days. *Acer campestre* was used as a shade-tolerant control. Plants were grown under standard greenhouse conditions in a random spatial arrangement under supplementary fluorescent lighting (12:12 hours light:dark) for seven days prior to treatments and all received the same quantity of water. After the initial period, five plants per species were transferred to the two enclosed environments within a protected environment for seven days.

Light environments were set at a 12:12h light:dark cycle and spectral composition was measured via a Skye Instruments (Llandrindod Wells, UK) R:FR meter. An LX-101 light meter (Labfacility; Feltham, Middlesex, UK) was used to quantify irradiance, a Decagon SC1 steady-state porometer (Decagon Devices; Pullman, Washington, USA) was used to measure stomatal conductance from the same leaves on each plant every two days and chlorophyll measurements were taken at the same time using a Konica Minolta 502-Plus SPAD meter.

Three dual-nodal lateral cuttings from each stock plant will be taken in early morning (08:00-09:30), sterilised for five seconds in 5% domestos solution (v/v) and washed in de-ionised water and placed in Braam paper plugs containing a 3:1 peat/vermiculite mix. Potted cuttings will be arranged on a misting bench with basal heating at 18^0C in a

RCBD arrangement (2 cuttings per treatment, therefore 20 cuttings in total over 10 sets). Five cuttings were enclosed in a 70ml test tube for 1hr in order to measure variations in ethylene evolution over time-course of rooting at 0h, 6h and 24h post-excision. 4ml of headspace ethylene from the cuttings was extracted by a luer-lock gas-tight syringe and stored in a pre-vacuumed 3.7ml Exetainer vial (Glatzel and Well 2008) and subsequently measured using GC-FID (as described in section **Methods; Extended Study**).

Fig.5. The environment chambers used for the red:far red experiment, equipped with high-intensity variable LED arrays used to manipulate the red:far red ratio.

Results

Initial Study

The soil moisture that stock plants are exposed to in the weeks leading up to cutting excision appears to play an important role in regulating the eventual rooting response (Figs. 6, 7, 8, 9 and 10). Both statistically significant reduced rooting percentage and mean root number in both *P. tremula* and *P. nigra* are the result of a 14 day exposure of stock plants to water deficits (150-300mV).

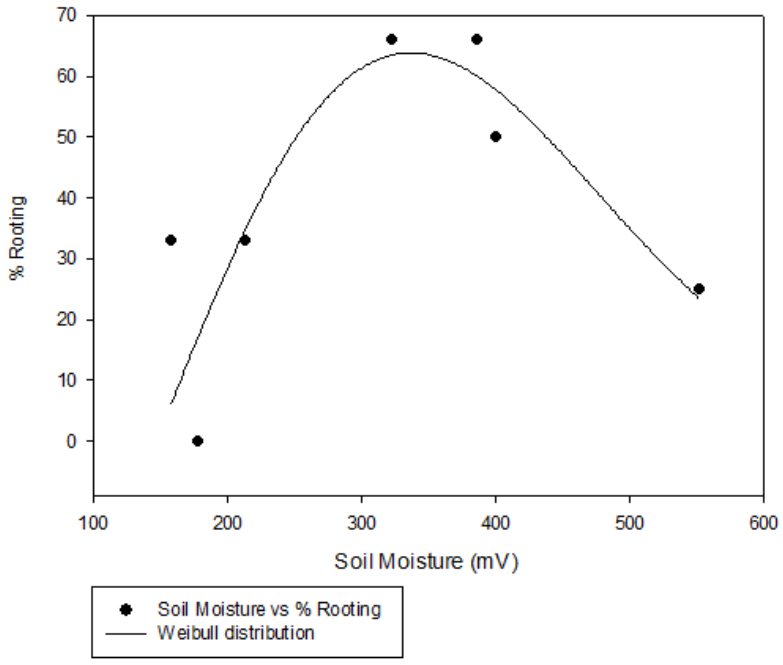

Fig.6. Distribution of rooting percentage produced by variation in the soil moisture exposure of stock plants of *Populus nigra.* Soil moisture levels are averages of the final four days of treatment. *(*n=3 or 4 per plant)

Even though there were a small number of total replicates, it was clear that there was a trend showing increased rooting in intermediate soil moisture ranges and reduced rooting in either low or high soil moisture (Fig.6), indicating an optimal range for eventual cutting success.

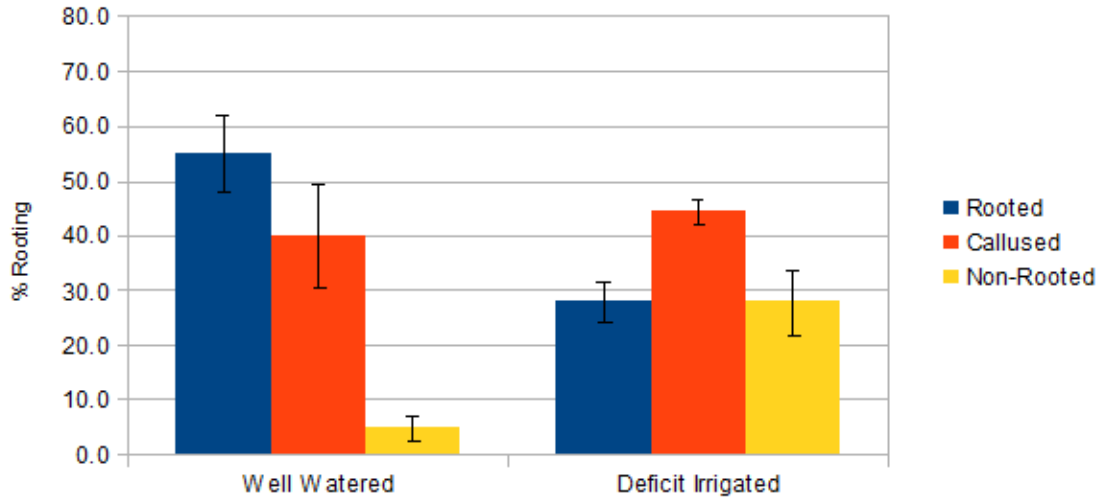

Fig.7. Mean rooting percentages for each soil moisture treatment in *Populus nigra* (n=24)

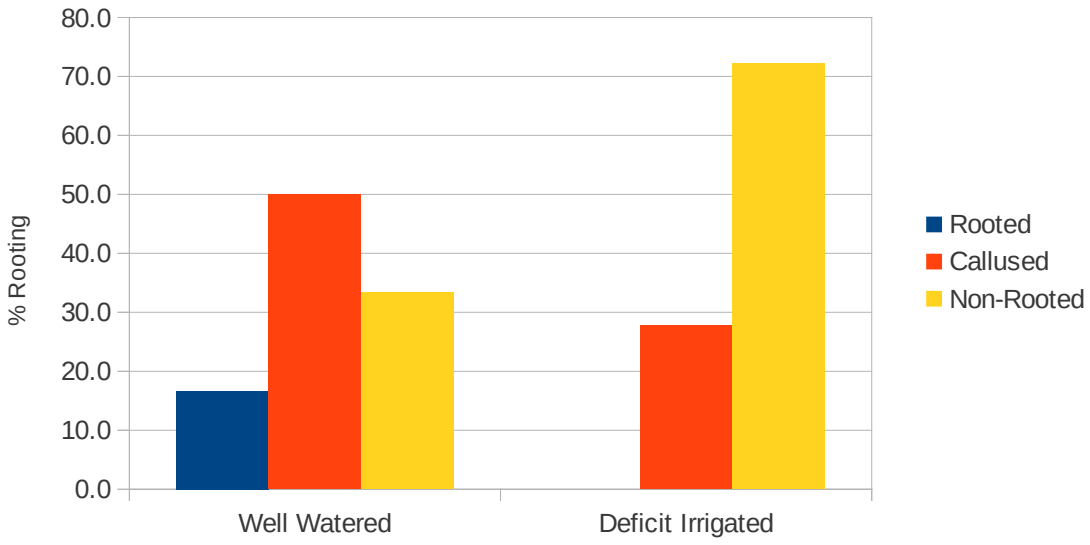

Fig.8. Mean rooting percentages for each soil moisture treatment in *Populus tremula* (n=24)

Both genotypes exhibit a significant reduction in rooting after imposition of deficit 55% to 28% in *P. nigra* (Fig. 7) and 16% to 0% in *P. tremula (*Fig. 8). In addition, the mean root number was reduced in *P. nigra* indicating that deficit treatments in stock plants produce stem tissues that are unable to produce acceptable quality liner material (Fig.9).

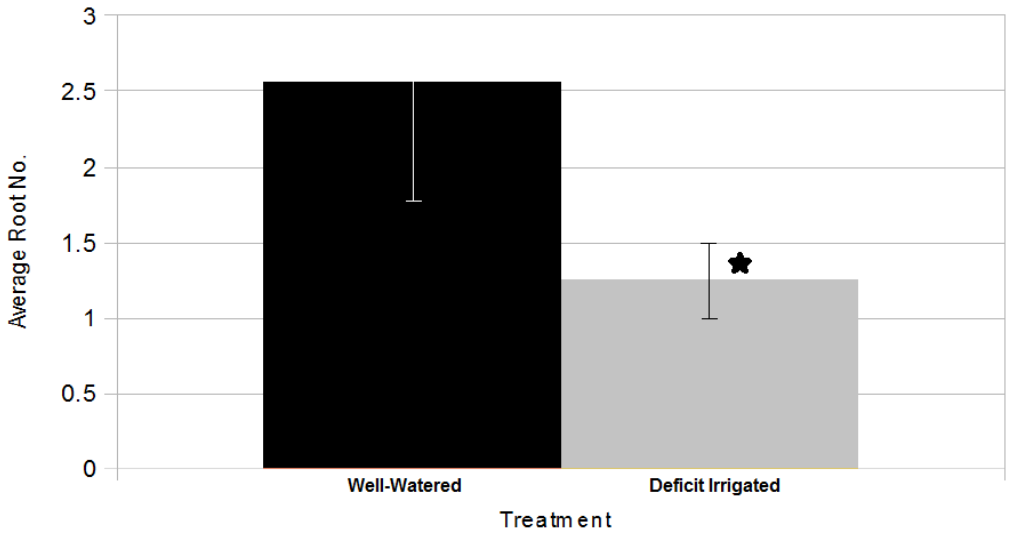

Fig.9. Mean root number for each soil moisture treatment in *Populus nigra.* (* denotes significant differences based on a student's t-test at 95% confidence limit), (n=24)

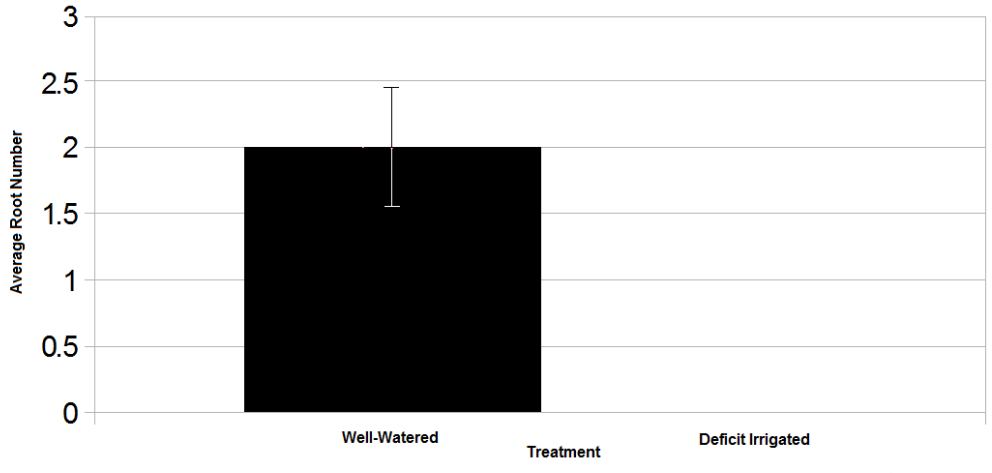

Fig.10. Mean root number for each soil moisture treatment in *Populus tremula.* (n=24)

Extended study using 4 species

At the time of writing, the data collection from the current experiment has only just been completed. Therefore, any discussion based on conclusive trends or explanations is not

possible at this stage. From the untransformed initial data however, there are differences in rooting with soil moisture, although these seem to be different for each species. Analysis of physiological, hormonal and biochemical data will enable us to explain these trends and will be discussed in the final report. A hypothetical model of how hormonal response to variation in soil moisture could produce such trends in rooting is shown below (Fig.11).

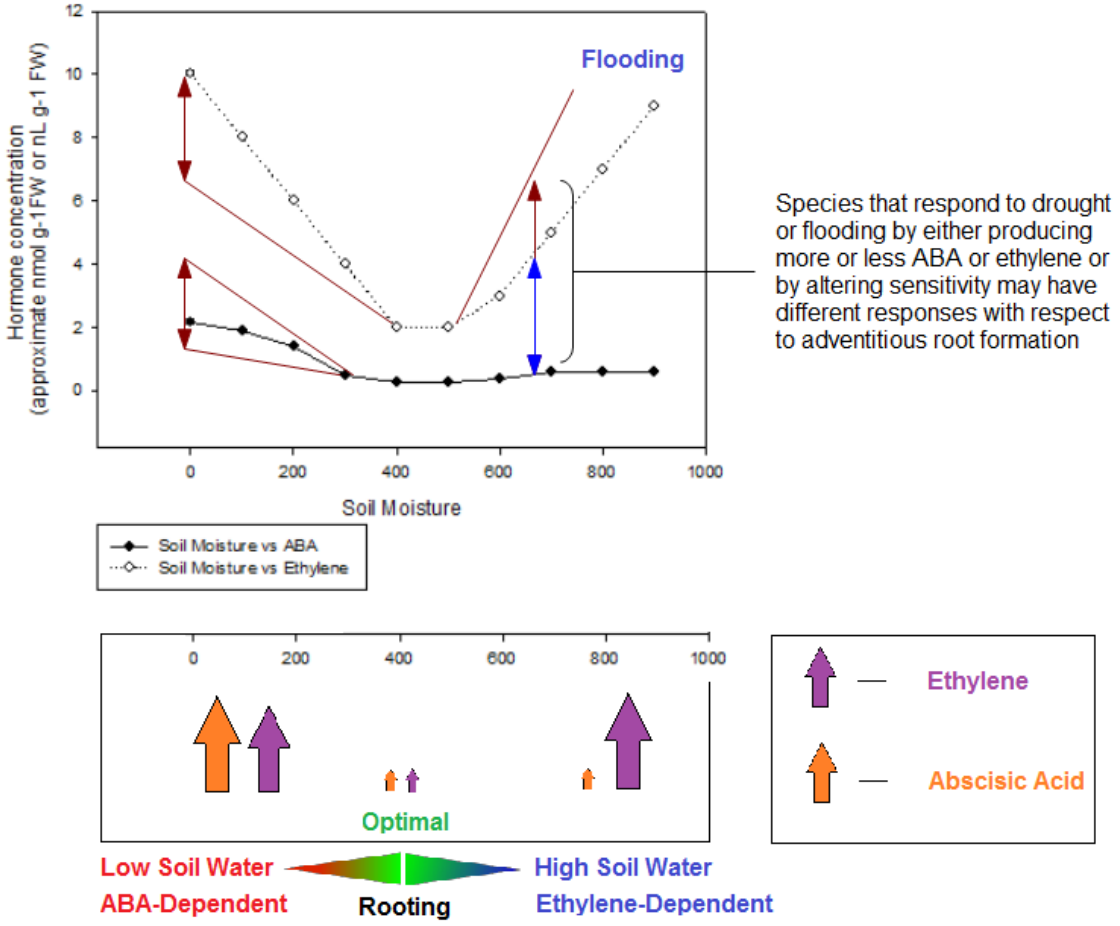

Fig.11. Hypothetical plot of ethylene and ABA variation with soil moisture showing how variations in the quantity of ABA or ethylene produced in response to low or high soil moisture could impact on final rooting. (Note that every media will have specific water holding and adsorption capacity based on particle size, charge and porosity)

Investigating the role of ABA in root induction

ABA-overproducing transgenics sp5 and sp12 produce less adventitious roots after 10 days compared to the wild-type (Fig. 12). Eventually, most of these cuttings root, so the actual phenotype is a delayed-response in ABA mutants, indicating that ABA is inhibitory early in the rooting process, which fits well with the hypothesis that it an antagonistic

interaction of ABA with ethylene after around 24 hours could limit root induction. Both transgenics produce about a third of the ethylene of the wild-type, but have about 10-times the ABA of the wild-type (Thompson, 2007).

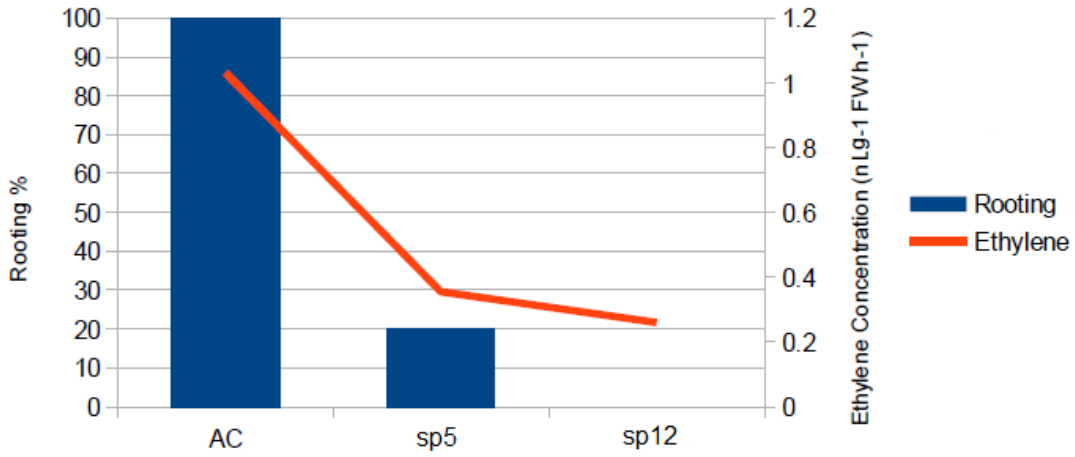

Fig.12. Rooting percentages at 10 days (blue bars) and ethylene emission at 24h after excision (red line) from ABA overproducing tomato mutants sp12 and sp5 and the corresponding wild-type 'Ailsa-Craig' (AC) (n=20).

Red:Far red ratio

The results indicate that physiological responses to low and high red:far red ratios are different between species. Interestingly, the shade-tolerant *Acer campestre* and in the apparently shade intolerant *Populus tremula* (Kull & Niinemets, 1998) increase chlorophyll content in both high and low red:far red ratio (Fig.13) and both increase stomatal conductance in red-enriched environments, but decrease slightly in far-red enriched (Fig.14). *Populus nigra,* however, had significantly reduced chlorophyll in far-red conditions (Fig.13) and reduced stomatal conductance in both conditions (Fig.14). It is probable that such responses would have implications not only for plant hormonal balance, but also on photosynthesis and carbon storage and partitioning, which are both important for rooting. It is interesting that *P. tremula*, not regarded as a shade-tolerant species, also increased chlorophyll content to compensate for potential reductions in intercellular carbon dioxide concentrations produced by a reduction in stomatal conductance.

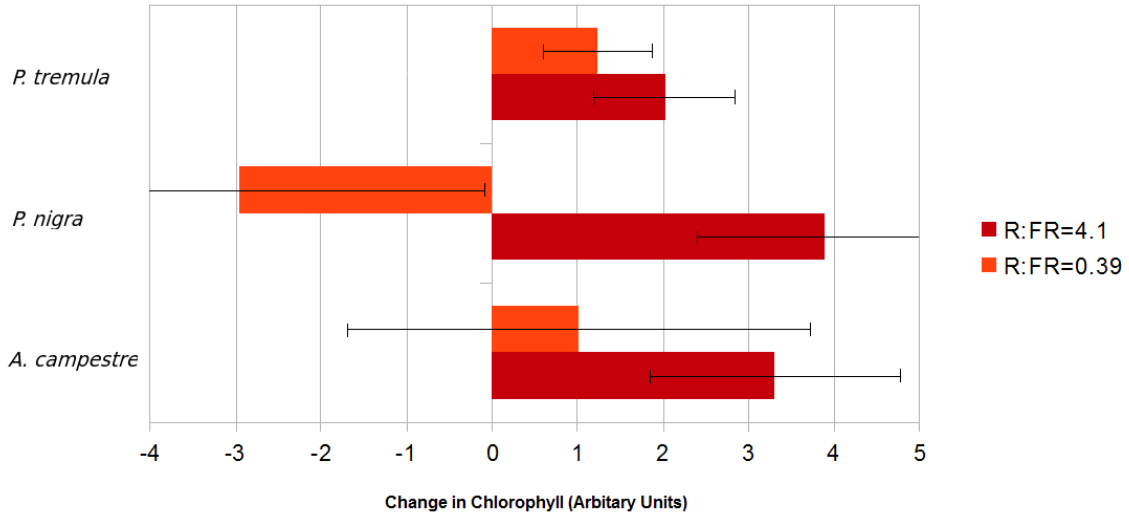

Fig.13. Change in leaf chlorophyll from the start to the end of the treatment period in red-enriched (Red) and far-red enriched (Orange) environments.

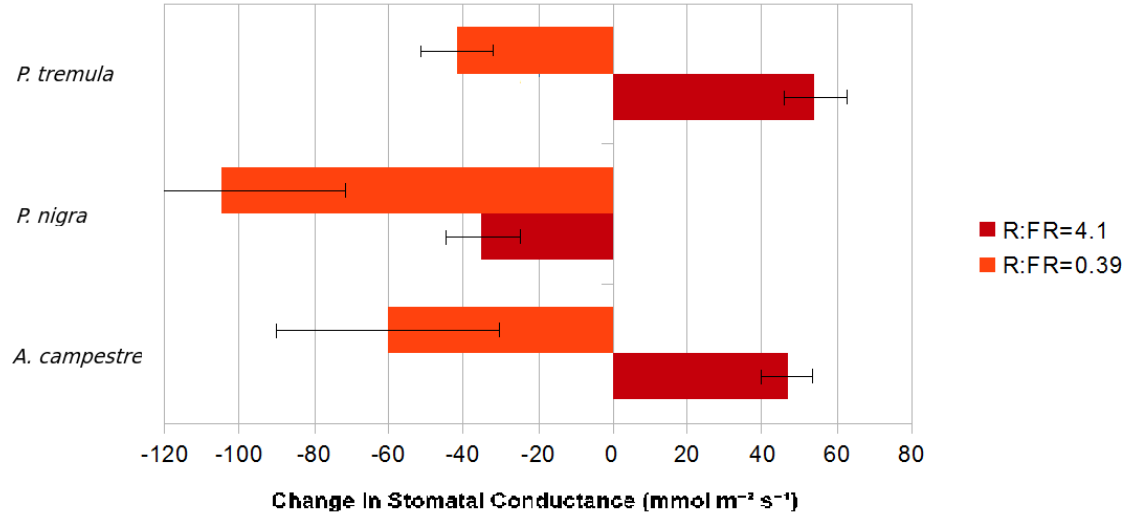

Fig.14. Change in stomatal conductance from the start to the end of the treatment period in red-enriched (Red) and far-red enriched (Orange) environments.

Conclusions

Variations in soil moisture availability may have a significant effect on the ability of subsequent cuttings to induce root formation (40-50% variation in rooting between treatments over selected time-periods). Environmental variation that produces ethylene and ABA concentrations that are outside of the optimal range for root induction may be responsible for differences in rooting potential of cuttings from genetically identical material. Strategies that attempt to limit the impact of this hormonal fluctuation on tissues

excised for cuttings may improve rooting.

Optimising irrigation management for species maybe one strategy in dealing with this with respect to soil moisture. Solutions do not have to be complicated or expensive; inexpensive systems based around commonly used irrigation schedulers coupled with soil moisture sensors can recoup their cost in very little time if cutting losses are reduced for high-value plant material.

Environmental factors in stock plants are important to the industry as highlighted by nurserymen when they talk about year-year climatic variability being responsible for variability in rooting and the quality of liners, but has yet to be comprehensively studied. It is expected that this project will be able to clarify the role that soil moisture plays in ARF the final year, especially with respect to differences in response in HONS species to various soil moisture treatments and deliver strategies to aid growers in monitoring and correcting for inadequate soil moisture.

Glossary

ABA; Abscisic acid: A plant hormone involved in the acclimation to stressful environmental conditions.

ACC: An ethylene precursor (converted to ethylene), produced endogenously via the action of the enzyme ACCsynthase

De-methylation: The process of removing methylation marks from genes in order to activate them.

Epigenetic: Changes that regulate the expression of genes, but do not involve a change in DNA sequence. May be transient or more stable.

ETp: Potential evapo-transpiration based on previous water usage.

Genotype: The underlying complement of genes present within a distinct group of individuals.

Leaf Plastochron Index: An index that can be used to classify the developmental stage of leaves from certain plants with a predictable leaf initiation and growth rate.

Meristematic Tissue: Groups of cells that are able to become any plant cell type.

Methylation: Refers to the process by which gene expression is repressed by addition of a methyl group to cytosine residues on DNA sequences.

Plastochron Index: A variation on the leaf plastochron index that can be used to classify the developmental stage of tissue from certain plants with a predictable leaf initiation and growth rate.

Stomatal Conductance: A measure of how 'open' plant stomata are and therefore how much water and gas-exchange (CO_2 and others) is occurring at the leaf level. Is an indicator of plant water stress as it generally decreases with decreased soil moisture.

Water Potential: A measure of the ability of water molecules to move to and from a given solution. A more negative water potential indicates the potential for water movement into the medium because of high solute concentrations, reduced water or an increase in electrostatic effects from the solid component of the media.

References

Baubec, T., Dinh, H. Q., Pecinka, A., Rakic, B., Rozhon, W., Wohlrab, B., von Haeseler, A., et al. 2010. Cooperation of multiple chromatin modifications can generate unanticipated stability of epigenetic States in Arabidopsis. *The Plant cell*, ***22:*** 34–47.

Boyko, A., & Kovalchuk, I. 2011. Genome instability and epigenetic modification-heritable responses to environmental stress? *Current opinion in plant biology*, **14***:* 260–266.

Chen, S., Wang, S., Altman, a, & Hüttermann, a. 1997. Genotypic variation in drought tolerance of poplar in relation to abscisic acid. *Tree physiology*, **17***:* 797–803.

Chinnusamy, V., Gong, Z., & Zhu, J.-K. 2008. Abscisic acid-mediated epigenetic processes in plant development and stress responses. *Journal of integrative plant biology*, **50:** 1187–95.

Chinnusamy, V., & Zhu, J.-K. 2009. Epigenetic regulation of stress responses in plants. *Current opinion in plant biology*, **12***:* 133–9.

Cohen, D., Bogeat-Triboulot, M.-B., Tisserant, E., Balzergue, S., Martin-Magniette, M.-L., Lelandais, G., Ningre, N., et al. 2010. Comparative transcriptomics of drought responses in Populus: a meta-analysis of genome-wide expression profiling in mature leaves and root apices across two genotypes. *BMC genomics*, **11***:* 630.

Dello Ioio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Gutierrez, L., Bussell, J. D., Pacurar, D. I., Schwambach, J., Pacurar, M., & Bellini, C. 2009. Phenotypic plasticity of adventitious rooting in Arabidopsis is controlled by complex regulation of

AUXIN RESPONSE FACTOR transcripts and microRNA abundance. *The Plant cell*, **21***:* 3119–32.

Ivanchenko, M. G., Muday, G. K., & Dubrovsky, J. G. 2008. Ethylene-auxin interactions regulate lateral root initiation and emergence in Arabidopsis thaliana. *The Plant journal : for cell and molecular biology*, **55*:*** 335–47.

Kim, D.-H., Doyle, M. R., Sung, S., & Amasino, R. M. 2009. Vernalization: winter and the timing of flowering in plants. *Annual review of cell and developmental biology*, **25***:* 277–99.

Klose, C., Büche, C., Fernandez, A. P., Schäfer, E., Zwick, E., & Kretsch, T. 2012. The mediator complex subunit PFT1 interferes with COP1 and HY5 in the regulation of Arabidopsis light signaling. *Plant physiology*, **160***:* 289–307.

Konieczny, R., Kępczyński, J., Pilarska, M., Cembrowska, D., Menzel, D., & Šamaj, J. 2009. Cytokinin and Ethylene Affect Auxin Transport-Dependent Rhizogenesis in Hypocotyls of Common Ice Plant (Mesembryanthemum crystallinum L.). *Journal of Plant Growth Regulation*, **28***:* 331–340.

Kull, O., & Niinemets, U. 1998. Distribution of leaf photosynthetic properties in tree canopies: comparison of species with different shade tolerance. *Functional Ecology*, **12***:* 472–479.

Li, C., Yin, C., & Liu, S. 2004. Different responses of two contrasting Populus davidiana populations to exogenous abscisic acid application. *Environmental and Experimental Botany*, **51***:* 237–246.

Lira-Medeiros, C. F., Parisod, C., Fernandes, R. A., Mata, C. S., Cardoso, M. A., & Ferreira, P. C. G. 2010. Epigenetic variation in mangrove plants occurring in contrasting natural environment. *PloS one*, **5 (4):** e10326.

Lisch, D. 2009. Epigenetic regulation of transposable elements in plants. *Annual review of plant biology*, **60***:* 43–66.

Liu, Q., & Chen, Y.-Q. 2009. Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. *Biochemical and biophysical research communications*, **384***:* 1–5.

Love, J., Björklund, S., Vahala, J., Hertzberg, M., Kangasjärvi, J., & Sundberg, B. 2009). Ethylene is an endogenous stimulator of cell division in the cambial meristem of Populus. *Proceedings of the National Academy of Sciences of the United States of America*, **106***:* 5984–9.

Morelli, G., Ruberti, I., Molecolare, B., Sapienza, L., & Moro, P. A. 2000. Update on Light Signaling: Shade Avoidance Responses. Driving Auxin along Lateral Routes. *Plant physiology*, **12***:* 621–626.

Negi, S., Sukumar, P., Liu, X., Cohen, J. D., & Muday, G. K. 2010. Genetic dissection of the role of ethylene in regulating auxin-dependent lateral and adventitious root formation in tomato. *The Plant journal : for cell and molecular biology*, **61*:*** 3–15.

Peng, H.-P., Lin, T.-Y., Wang, N.-N., & Shih, M.-C. 2005. Differential expression of genes encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis* during hypoxia. *Plant molecular biology*, **58***:* 15–25.

Quarrie SA, Whitford PN, Appleford NEJ,Wang TL, Cook SK, Henson IE, Loveys BR. 1988. A

monoclonal antibody to (S)- abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* **173**: 330–339.

Raj, S., Brautigam, K., Hamanishi, E. T., Wilkins, O., Thomas, B. R., Schroeder, W., Mansfield, S. D., et al. 2011. Clone history shapes *Populus* drought responses. *Proceedings of the National Academy of Sciences*, **108***:*12521–12526.

Ramirez-carvajal, G. A., & Davis, J. M. 2010. Identifying regulators of adventitious rooting. *Plant signaling & behavior*, **474***:* 281–283.

Ramírez-Carvajal, G. a, Morse, A. M., Dervinis, C., & Davis, J. M. 2009. The cytokinin type-B response regulator PtRR13 is a negative regulator of adventitious root development in Populus. *Plant physiology*, **150***:* 759–71.

Regier, N., Streb, S., Cocozza, C., Schaub, M., Cherubini, P., Zeeman, S. C., & Frey, B. 2009. Drought tolerance of two black poplar (Populus nigra L.) clones: contribution of carbohydrates and oxidative stress defence. *Plant, cell & environment*, **32:** 1724–36.

Ridolfi, A. M., Fauveau, M. L., Label, P., Garrec, J. P., & Dreyer, E. 2012. Responses to water stress in an ABA- unresponsive hybrid poplar (Populus koreana x trichocarpa cv . Peace), *New Phytologist* **134***:* 445–454.

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., et al. 2009. Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences of the United States of America*, **106***:* 4284–9.

Saze, H., Mittelsten Scheid, O., & Paszkowski, J. 2003. Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nature genetics*, **34***:* 65–9.

Sharp, R. G., Else, M. a., Cameron, R. W., & Davies, W. J. 2009. Water deficits promote flowering in Rhododendron via regulation of pre and post initiation development. *Scientia Horticulturae*, **120***:* 511–517.

Skylar, A., Sung, F., Hong, F., Chory, J., & Wu, X. 2011. Metabolic sugar signal promotes Arabidopsis meristematic proliferation via G2. *Developmental biology*, **351***:* 82–9.

Stamm, P., & Kumar, P. P. 2010. The phytohormone signal network regulating elongation growth during shade avoidance. *Journal of experimental botany*, **61***:* 2889–903.

Tao, Y., Ferrer, J.-L., Ljung, K., Pojer, F., Hong, F., Long, J. a, Li, L., et al. 2008. Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell*, **133:** 164–76.

Thompson, A. J., Andrews, J., Mulholland, B. J., McKee, J. M. T., Hilton, H. W., Horridge, J. S., Farquhar, G. D., et al. 2007. Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant physiology*, *143*(4), 1905–17. doi:10.1104/pp.106.093559

Wilkinson, S., & Davies, W. J. 2010. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, cell & environment*, **33***:* 510–25.

Wilmowicz, E., Kesy, J., & Kopcewicz, J. 2008. Ethylene and ABA interactions in the regulation of flower induction in Pharbitis nil. *Journal of plant physiology*, **165***:* 1917–28.

Appendix I

$$\text{Re-watering Quantity} = (M_i - M_{i+n}) \times \frac{\text{Required Moisture Ratio}}{\left(\frac{SM_{before} / SM_{after}}{\text{Required Moisture Ratio}}\right)}$$

Where; M_i is initial mass, M_{i+n} is current mass, Sm_{before} is the initial soil moisture and Sm_{after} is the current soil moisture level. The required soil moisture ratio accounts for the mean soil moisture for each range (i.e. 0.66 is used to calculate the mean soil moisture between 50% and 80% of carrying capacity of the media, which is generally around 720mV). The equation factors for differences in likely water usage between plants as well as incorporating data on moisture release from the soil. It also allows for easy adjustment of soil moisture content at different points in a drying cycle from plants of different size and slightly different soil dry mass. The change in mass (M_i - M_{i+n}) had been pre-transformed to reflect actual water transpired by adjusting for plant growth using the equation Y=0.975+0.112X that estimates biomass increase in *Populus* (Li, Yin, & Liu, 2004). The accuracy of these equations for estimating intended soil moisture was tested using by conducting a t-test on data from the expected and observed soil moisture for each plant.