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Factors affecting *in vitro* regeneration in the model tree *Populus trichocarpa* I. Medium, environment, and hormone controls on organogenesis

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Abstract
In preparation for a major GWAS (Genome Wide Association Study) of plant regeneration and transformation, a large number of factors were examined for their effects on indirect regeneration rate in diverse wild genotypes—seeking a high rate of regeneration, but also highly genetically variable and heritable treatments. Many of the factors examined have never before been reported on for their effects on callus, shoot, or root organogenesis in poplar (*Populus*). Stems had the highest regeneration potential, followed by petioles and leaves, with greenhouse grown explant sources superior to *in vitro* growth explant sources. Changes of ± 50% to Murashige and Skoog (MS) basal medium salts and micronutrients had a minor effect on regeneration. Many popular treatments that were evaluated also had little to no useful effect at the levels studied, including activated charcoal, ascorbic acid, silver nitrate, melatonin, serotonin, sucrose concentration, and lipoic acid. As a result of this wide exploration, treatment combinations that substantially elevated regeneration in diverse genotypes were identified, enabling GWAS.

Keywords Plant biotechnology · Forestry · *In vitro* regeneration · *Populus*

Introduction

Cottonwoods, white poplars, and aspens include approximately 30 species within the genus *Populus* (Eckenwalder *et al*. 1996); all are widely referred to as poplars. They are significant as a commercial tree for pulp and paper, are prized in construction for their lightweight and strong wood, and more recently have been cultivated as an energy crop for biofuel (Fillatti *et al*. 1987; Fladung *et al*. 1997; Bryant *et al*. 2020). In addition, they have become a model system for tree biotechnology (Ellis *et al*. 2010) because of their facile vegetative propagation to make clonal replicates, rapid growth in the greenhouse and field, high-quality diploid genome and related omic resources, and the facile capacity for genetic transformation and regeneration of individual genotypes.

The capacity for genetic transformation can vary widely within *Populus*. It was the first tree genus to be transformed, using *Agrobacterium* vectors and organogenesis (Fillatti *et al*. 1987; Busov *et al*. 2005). However, though individual genotypes, particularly within the white poplars of subgenus *Populus* (Fladung *et al*. 1997; Kutsokon *et al*. 2013) are readily transformed, Cottonwoods of sections Tacamahaca and Aigeiros (Eckenwalder *et al*. 1996) are much more recalcitrant and variable (e.g. Han *et al*. 1997; Yadav *et al*. 2009; Cavusoglu *et al*. 2011), requiring far more effort to develop regeneration and transformation (RT) systems (Holwerda *et al*. 2019). Among the difficult taxa is *Populus trichocarpa* Torr. and Gray, which contains many genotypes that are very difficult to regenerate transformants from using common organogenic methods. Thus, significant efforts have been put into developing effective regeneration and transformation systems for specific genotypes, including that of the reference genome individual Nisqually-1 (Ma *et al*. 2004; Song *et al*. 2006; Suzuki and Suzuki 2014; Li *et al*. 2017).

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The wide natural variation in RT among and within species, while generally known, has rarely been quantified or explored. Moreover, RT methods have often been developed for one or a few species, such as for Nisqually-1 as described above, and thus give limited insight about their general effectiveness. A greater understanding of how various medium and hormone components affect regeneration—studied in a diversity of genotypes and explant types—would inform overall transformation strategies. In addition, studies that seek to understand and make use of the high genetic diversity in regeneration and transformation capacity that is reported in nearly every plant species, such as through GWAS (genome wide association study), requires a broad understanding of how various treatments affect the rate of regeneration in a large sample of genotypes. GWAS studies of *P. trichocarpa* for the related *in vivo* traits of shoot and root regeneration were recently published (Nagle *et al*. 2022a, b). In particular, treatments that maximize the expression of genetic vs. environmental sources of variation (broad-sense heritability for clonal species: H^2) need to be identified, as those are generally the basis of the most effective genetic association analyses. For example, Tuskan *et al.* (2018) used a treatment that gave high H^2 (0.67) to identify several candidate genes for control of callus regeneration in a pilot study of *P. trichocarpa*.

Many transformation studies employ indirect regeneration—where organs (usually shoots) are induced after a period of callogenesis thought to enhance totipotency and allow antibiotic selection prior to shoot meristem differentiation (Bhatia *et al*. 2015). For this reason, most of the studies employed indirect methods. However, direct regeneration can be highly effective in specific systems, including for the recalcitrant Nisqually-1 (Li *et al*. 2017). Thus, variation in direct and indirect regeneration responses among genotypes was briefly compared. Explants that were placed directly onto a highly effective SIM (shoot induction medium) were compared to that from placement on a widely used type of CIM (callus induction medium) prior to SIM.

Basal media (BM) types are rarely studied with respect to regeneration rate. The large majority of studies employ Murashige and Skoog (1962) media for *in vitro* regeneration (Aggarwal *et al*. 2015) because of its balanced composition that is suitable for a wide variety of plant species, with variations of its major salts rarely considered. Therefore a partial factorial study of 36 different variations of the major salts that compose MS media was conducted, which was then partially replicated after the most promising media were identified.

A number of other factors widely known to affect regeneration rate were also briefly examined, including auxin and cytokinin types and concentrations, the use of the recently popular melatonin and serotonin (Erland *et al*. 2015, 2016); and the use of several popular approaches to reduce plant oxidative stress and associated ethylene signaling (*e.g.,* Dan *et al*. 2009), as well as chemicals to mitigate microbial contamination (a challenging problem when using *in vivo* explants). PPM (Plant Preservative Mixture, plantcelltechnology.com) and benomyl are both broad-spectrum fungicides/biocides commonly used to control contamination in plant tissue culture (Bollen and Fuchs 1970). The overall flow and logic for the experiments is outlined in Fig. 1.

Reported in this article are the results of 12 different experiments involving 170 treatments, with each experiment using two to 20 genotypes and two to three replicates per treatment, for a total of 42,648 tested explants. Here, results on rates of regeneration, averaged over genotypes, are presented. Treatments that substantially elevate the rates of regeneration are also identified. Analyses of genetic control, H^2, and genetic interactions with treatment factors are presented in a companion paper (Ma *et al*. 2022). To avoid contamination, *in vitro* explants were often used in early experiments; however, because the planned GWAS experiments employed *in vivo*-derived plants (because of the scale of the work), *in vitro* studies were often repeated with *in vivo* explants, and later experiments focused predominantly on *in vivo* explants. Despite the recalcitrance of this species to common methods of regeneration and genetic transformation, treatments that enabled high levels of successful regeneration in many genotypes were identified, and as shown in

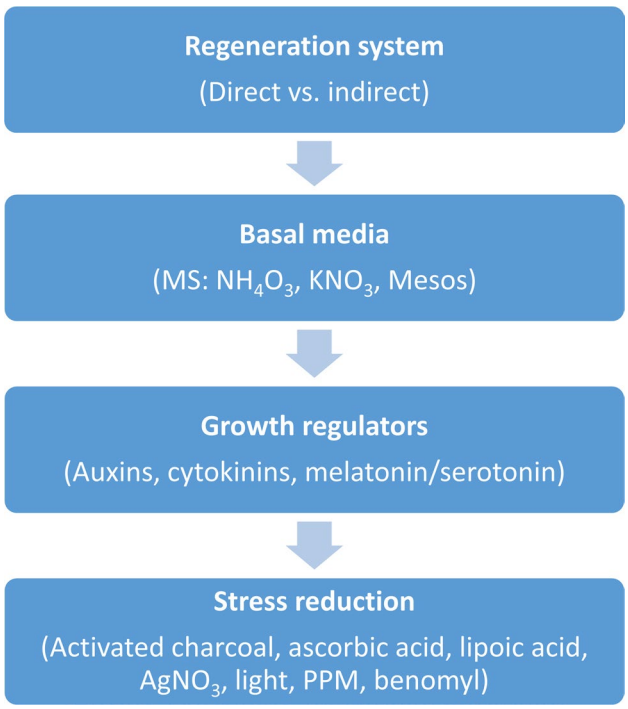

Figure 1. Overview of sequence and rationale for experiments.

a companion paper (Ma *et al*. 2022), these regeneration responses were highly heritable.

Materials and Methods

The experimental structure is summarized in Table 1; additional experimental details are provided in supplemental Table S1 (experimental details), S2 (basal medium salts and micronutrients for experiment E2), and S3 (basal medium designations and experimental means for all traits and experiments as heat-maps). Apart from experiment E1, where direct and indirect organogenesis were compared, all other experiments used an indirect system. For each experiment, up to 20 different genotypes of wild *P. trichocarpa* from the Pacific Northwest USA and nearby Canada were used. Genotypes used are part of a GWAS association population assembled by Oak Ridge National Laboratory (bioenergycenter.org/besc/gwas). Dormant branches were collected from a clone bank in the field in Corvallis, Oregon then rooted in the greenhouse, from which stem, leaf, or petiole explants were taken and established *in vitro* (harvested explants classified as *in vitro*) or used directly after sterilization (the latter because the approximately 1,300 genotypes employed for GWAS work made it infeasible to maintain all of the cultures *in vitro*). Greenhouse materials were sterilized *via* 3 to 5 min in 70% ethanol and 10 to 15 min in 20% bleach (8.25% sodium hypochlorite) for leaf, stem and petiole, rinsed 4 times in sterile water, then 6 mm leaf discs were placed adaxial side up and 3 to 4 mm stem and petiole explants (classified as *in vivo* explants) on Petri dishes containing varying combinations of basal media and other additives.

All explants were then allowed to grow for several wk, with callus traits being scored at after about 3 wk on CIM in the dark and shoot/root traits scored in the light approximately four wk after that (refer to individual experiments for specifics). All explants were cultured in the growth chamber at 25 °C with fluorescent lighting at 40 µmol m^{-2} s^{-1} with a 16 h photoperiod, except E8, where explants were cultured in fluorescent and LED growth chamber lights at 25 °C with the same light intensities (40 µmol m^{-2} s^{-1}). RGB images of Petri dishes

Table 1. Summary of experimental treatments studied. Additional details on experiments are in Tables S1 and S2. Most experiments included *Populus trichocarpa* Torr. and Gray leaf, stem, and petiole explants

Experiment ID	Factors studied	Number of genotypes	Notes
E1	Direct vs indirect regeneration	20	CIM23[a] to SIM23[b] vs. SIM23 directly, SIM23 moved to lower TDZ concentration after 3 wk, *in vivo* explants
E2	36 variations of CIM, then SIM23	2	CIM1-CIM36[c]; then SIM23 as described in supplementary materials, *in vitro* explants sources
E3	Further tests of 5 SIM-CIM combinations	17, 6	CIM11, 19, 23, 24, 28; then SIM11, 19, 23, 24, 28, *in vitro* (6) & *in vivo* (17) explants
E4	Varying sucrose levels	4	CIM 19_1[d],23 to SIM 19,23 *in vitro* explants
E5	Various auxins and concentrations	20, 6	CIM 19_1 to SIM 19, *in vitro* (6) & *in vivo* (20) explants
E6	Various auxin/cytokinin combinations	16	CIM19_1, SIM19, *in vivo* explants
E7	Melatonin and serotonin concentrations	19	CIM19_1 then SIM19, *in vivo* explants
E8	Fungicide concentrations, PPM & benomyl	8	CIM19_2[e] then SIM19, *in vivo* explants
E9	Lipoic acid concentrations	16	CIM19_2 then SIM19, *in vivo* explants
E10	Activated charcoal, ascorbic acid	16	CIM19_2 then SIM19, *in vivo* explants
E11	$AgNO_3$ ethylene inhibitor	16	CIM19_2 then SIM19, *in vivo* explants
E12	Light spectrum and intensity	4	CIM19_1 then SIM19, *in vivo* explants

Notes:

[a]CIM23 = callus induction medium, composition = Full strength Murashige and Skoog (MS) salts (Cassion Labs MSP01-10LT). with 1.86 mg L^{-1} 1-Naphthaleneacetic acid (NAA) and 1 mg L^{-1} 2-isopentenyladenine (2ip) and 3% sucrose

[b]SIM23 = shoot induction medium, composition = Full strength MS salts with 0.132 mg L^{-1} TDZ (Thidiazuron) and 3% sucrose

[c]CIM1-36 described in supplementary materials, varied salts and 1.86 mg L^{-1} NAA and 1 mg L^{-1} 2ip and 3% sucrose

[d]CIM19_1 = callus induction medium, composition = modified MS salts with 1.86 mg L^{-1} NAA and 1 mg L^{-1} 2ip and 3% sucrose for E3-E7 and E12

[e]CIM19_2 = callus induction medium, composition = modified MS salts with 0.01 mg L^{-1} 2,4-Dichlorophenoxyacetic acid and 3% sucrose for E8-E11

[f]SIM19 = shoot induction medium, composition = modified MS salts with 0.132 mg L^{-1} TDZ and 3% sucrose

were visually scored for several parameters, as explained below under quantitative analysis.

Quantitative Analysis In all experiments, growth data were measured manually based on Petri dish images with the following scoring system:

- Callus formation: 0 for no callus, 1 for callus < 1 mm, 2 for callus between 1-3 mm, and 3 for callus > 3 mm.
- Root formation: 0 for no root, 1 for 1–3 roots, 2 for 4–5 roots, and 3 for more than 5 roots (only roots longer than approximately 1 mm were counted)
- Shoot formation: 0 for no shoots and 1 for at least 1 shoot (only shoots longer than approximately 3 mm were counted)
- Shoot number: The actual number of shoots counted for each explant

The Petri dish (plate) was considered as the experiment unit (replicate) with explants within plates as pseudo-replicates. Growth data were therefore summarized on a per plate basis—which should also enhance their approximation to a normal distribution—for the following six traits that represent the development of the explants:

- Proportion of explants that form callus per plate for each explant type (denoted by "proportion of explants with callus")
- Proportion of explants that form shoots per plate for each explant type (denoted by "proportion of explants with shoots")
- Proportion of explants that form roots per plate for each explant type (denoted by "proportion of explants with roots")
- Average size of callus per plate for each explant type (denoted by "callus size")
- Average number of shoots per plate for each explant type (denoted by "shoot number")
- Average number of roots per plate for each explant type (denoted by "root number").

To visualize treatment effects, boxplots of each trait versus the treatment are presented for each explant type. These boxplots present the effect of different treatments on the growth of different explant types averaged over genotypes as biological replicates. The variation among genotypes was graphed and statistically analyzed using ANOVA, and presented in a companion paper (Ma *et al*. 2022). Scatter plots of these average traits are presented to show the correlation between different traits as well as between different explant types.

The data in experiment E2 to screen the 36 basal media were also analyzed with a response surface method to explore the relationship between the concentrations of nitrate and meso-nutrient solutions. A full second-order response surface model was used to try and determine the optimal concentrations. The results are presented as response-surface contour plots in which the optimal solution is represented as the center of the contours when the model was able to identify an optimal solution.

To gain insight into the replicability of regeneration phenotypes across experiments that were conducted at widely different times, subsets of data that utilized the same genotype/treatment combinations were extracted for analysis; ANOVA and graphical results are presented in Ma *et al*. 2022.

Results and discussion

E1: Direct vs. Indirect Regeneration The first experiment sought to assess the rates of shoot regeneration under an indirect versus a direct regeneration system, using hormones commonly employed in the Strauss laboratory for this purpose in poplars. As detailed in a companion paper (Table S1 in Ma *et al*. 2022), treatment effects were statistically significant for both callus and shoot regeneration, as well as for treatment x explant interaction. When averaged over all 20 genotypes, the rate of callus formation was, as expected, much higher with the indirect system (on average, greater than 80% of explants with callus) compared to the direct system (less than 20% of explants with callus) (Figure S1 E1, Table S3), and both leaf and petiole explants behaved similarly. Root regeneration was observed in 40% of explants undergoing indirect regeneration from leaves, whereas only a handful of plates showed any root regeneration from petioles (mean of 9%); no roots were seen under direct regeneration (Figure S2 E1, Table S3). The value of the indirect system was most striking when shoot regeneration was measured after 3 wk on SIM with TDZ (0.22 mg L^{-1}), then 3 wk on a reduced TDZ containing medium (0.022 mg L^{-1}), where the mean proportion of explants with shoots was 39 to 45%, compared to a rate of 7 to 23% from direct regeneration (Figure S3 E1, Table S3). Leaves had somewhat higher mean callus and shoot regeneration responses than petioles in the indirect system (89 vs. 82% and 45 and 39%, respectively), but the ranks were reversed in the direct system (9 versus 19% and 7 versus 23%, respectively) (Table S3). As expected, when the means of the three replicates per treatment were graphed, the results showed opposing effects for direct versus indirect treatments for both callus and shoot regeneration (Figure S4 E1 and S6 E1).

These results suggest that callus growth during the auxin-rich indirect regeneration treatment increases the

competency or number of *P. trichocarpa* callus cells that can respond to shoot regeneration-inducing hormones, a result that has been observed to various degrees in other poplars (Jehan *et al*. 1994; Zhou *et al*. 2012) and many other plant species (de Oliveira *et al*. 2011) though exceptions have also been noted (Yadav *et al*. 2009; Biswas *et al*. 2012). However, as shown in a companion paper (Ma *et al*. 2022), for many genotypes a CIM treatment is not needed for high rates of shoot regeneration; if transgenic cells can still be selected and regenerated, this would speed regeneration of transgenic shoots and reduce somaclonal variation (Jehan *et al*. 1994).

E2: Basal Media Before studying hormone and other treatments to optimize regeneration rate, basal media composition was first examined, specifically the major nitrogen salts and micronutrient levels. Regeneration responses to various hormones can be strongly affected by basal medium chemistry (Phillips and Garda 2019). Inspired by Niedz and Evans (Niedz and Evens 2007), a factorial design was used to vary their levels 1.5 to 0.5-fold compared to MS medium levels (Table S2), giving a total of 36 medium combinations that were tested on *in vitro* explants from two genotypes. The same CIM hormones were used as in experiment E1, but a modified SIM (lower TDZ) was employed. Treatments (basal media) were a statistically significant source of variance for all traits (Ma *et al*. 2022), though accounted for a modest amount of variance (less than 10% for all traits except callus size). The treatment x explant type interactions were significant only for the callus traits and root proportion, but accounted for only 1% of the observed variance.

The level of callus formation was uniformly high, with all treatments being near to or at 100% regeneration from all explant types, though stems tended to produce larger calluses than the other explants (Table S3, Figure S1 and S2, E2). The rate of root formation was greatest from leaves followed by petioles then stems, with basal media affecting the rates for petioles substantially, with regeneration rates varying from zero to 100% between media (Figure S3 and S5 E2). The rate of shoot regeneration was strongly affected by medium type, ranging from zero to 100% (Figure S4 E2). Shoot number was generally well below one shoot per explant (mean of 0.9: Table S3), though variation among media, and the maximum number of shoots per explant for the best media, was highest for stems (Figure S6 E2). Correlations among regeneration responses for the explant types for a common trait (Figures S7-S12 E2), or among traits averaged over explants (Figures S13-S16 E2), were positive but all low, suggesting that regeneration responses were similar among media and often influenced by random variation, which is also not surprising given that regeneration rates per explant were generally low.

When overall correlations among traits, considering all the basal mediaum and explant types, were viewed with scatter matrices, it was clear that associations were consistently positive but weak (Figures S13-S16 E2). Callus size and shoot number per explant, and shoot number and root number per explant, had a correlation of about 0.1, whereas the association was approximately twice as strong for proportion of explants with root vs. those with shoots, and proportion of explants with shoots vs. those with callus. Thus, the results suggest that basal media and explants have tendencies to favor regeneration in general, and were expressed in several traits. Most importantly, conditions that favored callus production tended to favor shoot production, and conditions that favored root production also favored shoot production.

When the response surfaces for all the factors combined were modeled, optimal combinations of factors were not clearly defined, and often differed among explant types (Figures S17-S25 E2), even with this large experiment. For example, though intermediate levels of KNO_3 were consistently near to optimal for shoot regeneration, the levels of NH_4NO_3 that were close to optimal varied widely among explant types, with stems benefitting from higher levels than leaves, which were higher than petioles (Fig. 2). Of

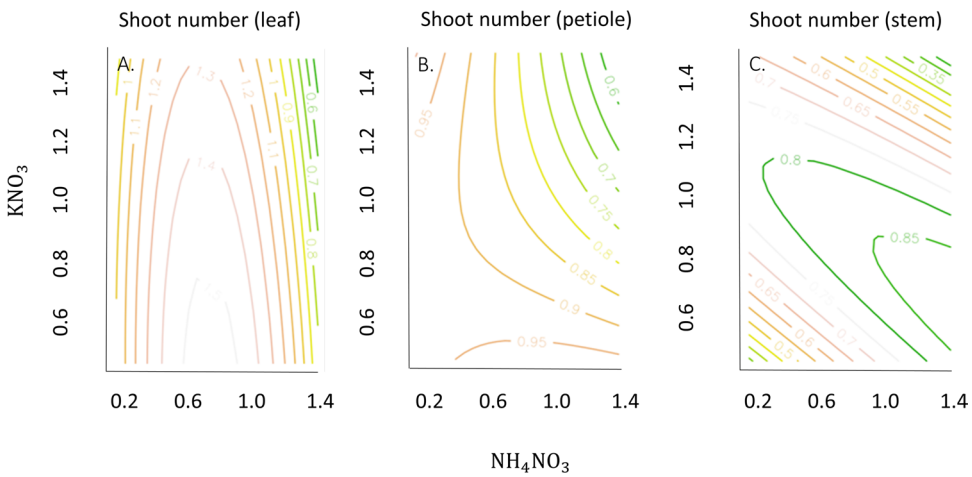

Figure 2. Modeled optima for KNO_3 in relation to NH_4NO_3 in basal media. Data shown for *Populus trichocarpa* Torr. and Gray shoot number from (*A*) leaf (*B*) petiole and (*C*) stems from experiment E2. Values on axes are multiples of standard MS media concentrations (1X NH_4NO_3=20.625 mM; 1X KNO_3 = 18.812 mM).

the 27 combinations that were graphically examined, only two showed a clear optimum, the combinations of mesonutrients and NH_4NO_3 for shoot number from leaf explants (Figure S23 E2) and the combination of KNO_3 and NH_4NO_3 for root number from stem explants (Figure S22 E2). As a result of these experiments, five highly promising media with respect to rate of shoot regeneration were chosen, for which results were replicated (see below), including examining responses of *in vivo* materials that were planned for use in GWAS studies.

E3: Replication to Select Final Basal Media When *in vitro* materials were again used, but here with 6 rather than just two genotypes as in experiment E2, the top five basal media selected from experiment E2 all performed well again, and similarly to each other (Figures S1-S6 E3a). Basal media were statistically significant sources of variance, though accounted for less than 5% of variance (Table S1 in Ma *et al*. 2022). Explant types were also a significant source of variance, accounting for 4 (shoot number) to 40% (root number) of variance. Basal medium by explant interactions were non-significant and only about 1% of variance. Averaged over genotypes, stems were the most regenerable in terms of callus size and frequency, and only leaf explants gave substantial root formation. Stem explants have also been reported to be highly regenerable in other species; for example, in wild apples stem explants showed higher callus induction and a low death rate on SIM supplemented only with BA as compared to leaf explants. Callus induction from stem explants was also faster than induction from leaf explants, and showed the highest number of regenerated shoots per explant (Zhang *et al*. 2020).

Averaged over genotypes, the correlations among the three explant types for the various basal media and replicates thereof were nearly all positive though modest (Figures S7-S12 E3a), suggesting the most regenerative conditions had positive effects expressed in all the explant types. The one exception was root proportion between stems and leaves, whose correlation was weakly negative (Figure S9 E3a). When correlations among all the treatments and explant types were combined to view general trends, shoot and callus had modest positive associations (Figures S13-S14 E3a), suggesting callus growth promotes or diminishes shoot regeneration. However, shoot and root relationships were negative, a result of the much higher root regeneration rate from leaves combined with its lower rate of shoot regeneration (Figures S15-S16 E3a).

In order to reduce the large amount of resources that would be needed to maintain the many thousands of genotypes and replicate *in vitro* materials needed for GWAS, *in vivo* explant materials were employed with the same five basal media. The patterns were similar but different in a few ways from results with *in vitro* explants (Fig. 3 & Figures S1-S5 E3b). Main effects and most interactions were statistically significant (Table S1 in Ma *et al*. 2022). Averaged over 17 genotypes, stem explants again gave the highest callus and shoot regeneration, also with modest differences among basal media. Root regeneration was again highest from leaves (Figure S4 E3b, S6 E3b, S16 E3b), but not as high as seen with *in vitro* explants, and some root regeneration was observed from stems, but only for BM11 and BM19 (Figure S4 E3b). Total shoot and callus regeneration tended to be higher with *in vivo* vs. *in vitro* regeneration (e.g., shoot proportion from stems was approximately 50% vs. 20%, respectively, and callus size class was nearly double). The striking differences in regeneration between *in vitro* and *in vivo* materials, as well as the high genotype dependence in response to regeneration treatment, is illustrated in Fig. 4 (experiment E5). This difference was also seen in genetic transformation of *P. trichocarpa* Nisqually-1 (Song *et al*. 2006), where about 46% of stem segments from greenhouse plants produced putative calluses, but no callus was produced from stem explants derived from *in vitro* plants.

The correlations among explant types for the regeneration traits was again moderate and consistently positive (Figures S7-S12 E3b), with root regeneration lowest or highly non-linear due to only leaves having substantial regeneration. Considered over all media and explant types, shoots and callus were again positively correlated, but roots and shoots had very low correlations due to the combination of very high root regeneration and low shoot regeneration from leaf explants (Figures S13-16 E3b). Although medium was a statistically significant source of variance for all traits, it generally accounted for less than 5% of the phenotypic variance; media 24 and especially 28, which had the highest level of NH_4NO_3 tested (increasing above MS levels), had somewhat reduced callus formation, but shoot and root development were comparable in all tested media. The quantity of ammonium nitrogen and total nitrogen are much higher in MS medium than in the majority of plant media used. For example, oil palm plantlets regenerated on Y3 and B5 media showed marked differences in most growth parameters compared to MS medium (Onyeulo *et al*. 2018). When studying *in vitro* plant regeneration from mature embryo explants of *Jatropha curcas* L., B5 medium was superior to MS medium (Comfort *et al*. 2017). Low concentrations ($\leq 1\times$) of ammonium nitrate and iron has also been seen to result in increased shoot regeneration across genotypes during micropropagation (Reed *et al*. 2013).

E4: Sucrose Level Two of the best basal media from experiment E4 were used, numbers 19 and 23, to study whether modified sucrose levels (1.5 to 3.0%) would affect regeneration rate. The main effects of sucrose treatment were statistically significant for all but the callus traits (Table S1, Ma *et al*. 2022). The highest levels of sucrose tended to give the best rate of shoot

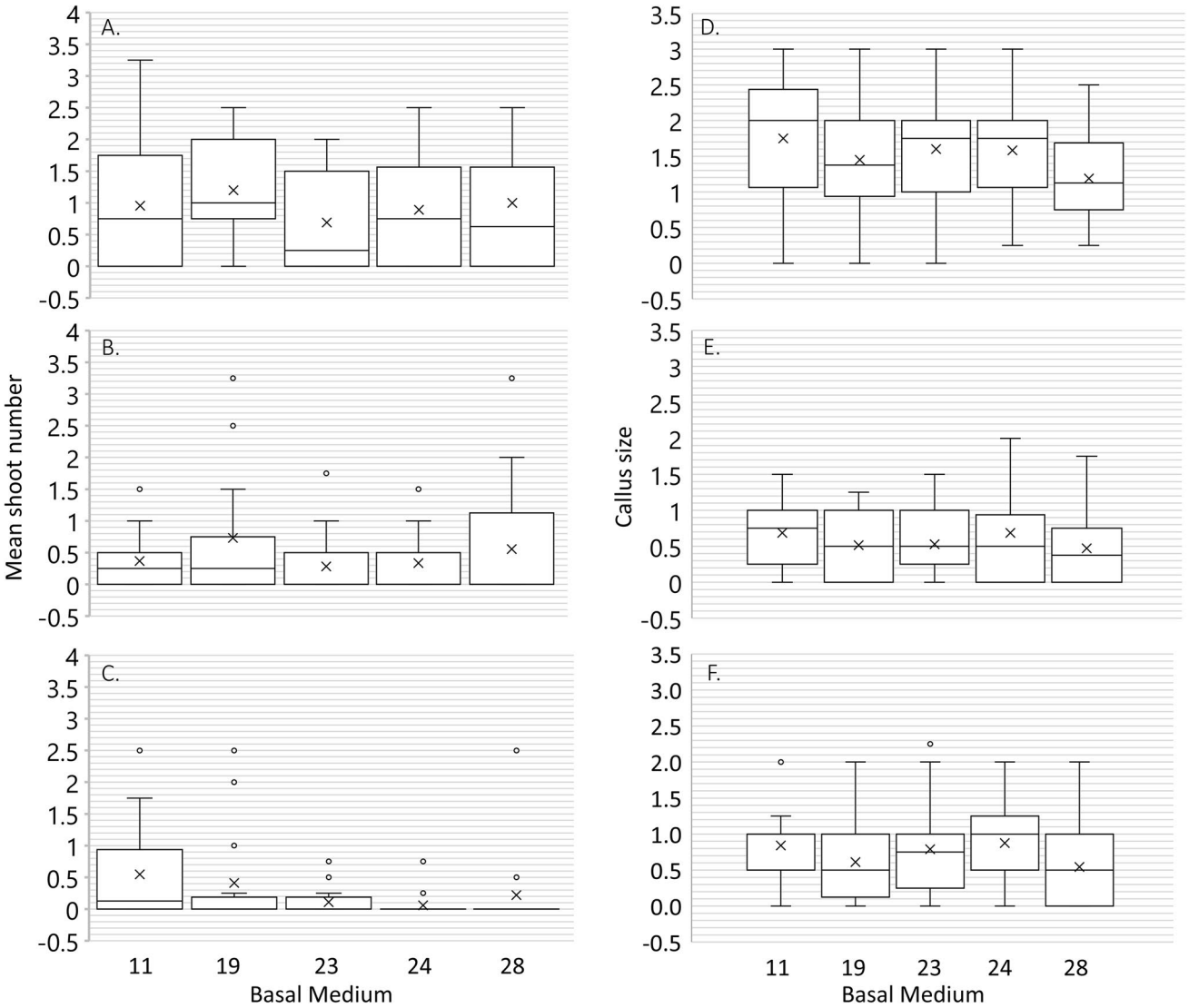

Figure 3. Effects of varied MS basal medium on generation of *Populus trichocarpa* Torr. and Gray callus and shoots. Box plots of callus size (*right column*) and shoot number (*left column*) averaged over genotype for (*A*, *D*) stem, (*B*, *E*) petiole, and (*C*, *F*) leaf explants from experiment E3 with *in vivo* explants. Basal medium compositions are given in Table S2. "x" marks show the mean, and *horizontal lines* the median. The *boxes* themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

regeneration, an effect that was most striking in stem explants (Figures S1 & S6, E4). The effects on callus were complex, and there was a tendency for root formation from leaves to be reduced as sucrose was increased (Figures S2-S5, E4).

Responses were weakly but usually positively correlated among explant types, with the physiologically similar stem and petiole showing the strongest association in four of five scatter matrices (Figures S7-S11, E4). Since the stem and petiole explant types had similar responses, but are distinctly different from leaf, this will inform how experiments are structured (e.g., to conduct GWAS on test stem and petiole explants in the same sucrose, but leaf explants in separate conditions).

Although not observed in this study, high concentrations of sucrose can inhibit plant growth, slow photosynthetic pathways, and cause other developmental effects (Bhatia *et al*. 2015; Freitas *et al*. 2015; Thompson *et al*. 2017). A low concentration of sucrose (29 mM) (1%) strongly reduced the regeneration ability from leaves of *Nipponanthemum nipponicum*, perhaps indicating inadequate energy resources. A high sucrose concentration of 147 mM (5%) decreased regeneration as well, suggesting an osmoticum shock which blocked differentiation. A sucrose concentration of 88 mM (3%) gave the highest rates of shoot regenerating explants and a high number of shoots per regenerating explant (Tosca *et al*. 1999).

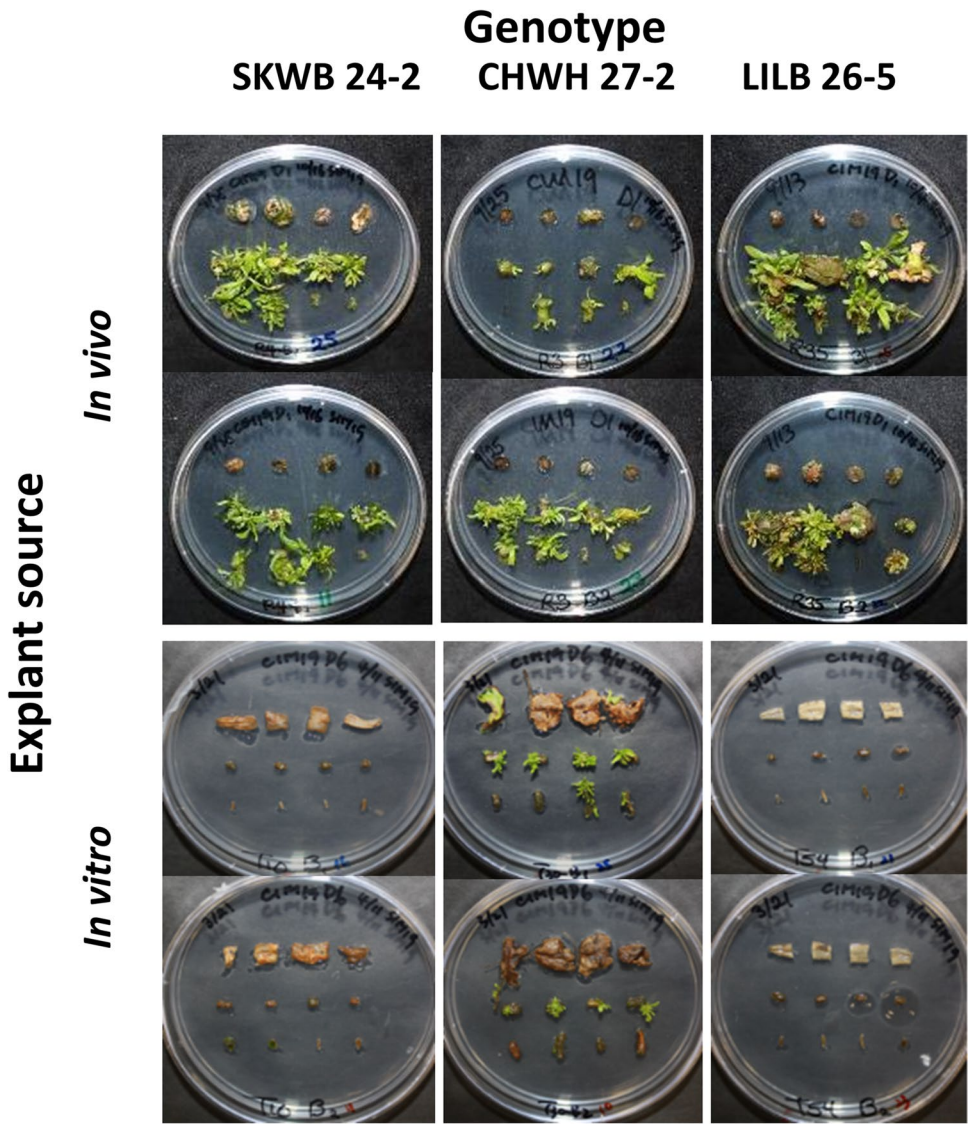

Figure 4. Variation in rate of indirect *Populus trichocarpa* Torr. and Gray shoot regeneration in relation to genotype, source tissue, and explant type. Data from experiment E5 (*in vitro* vs. *in vivo* explant sources). Indirect organogenesis was used with callus induction for 21 d on CIM19 with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.01 mg L^{-1} and shoot induction for 42 d using SIM19 with thidiazuron (TDZ) at 0.132 mg L^{-1}. There are two plates shown for each genotype and source, coming from different experimental blocks. The three *horizontal rows* of explants in each Petri dish, from *top* to *bottom*, are leaf discs, stems, and petioles.

E5: Auxin Types and Levels Auxin effects both with *in vitro* and *in vivo* derived explants were studied. Treatment effects were statistically significant for all traits and were generally the highest source of variance (Table S1, Ma *et al*. 2022). Overall, *in vitro* explants showed weaker regenerative responses and higher rates of necrosis when subjected to the same hormone treatments, and genotypes responded differentially (e.g., Fig. 4). Another example of the strong auxin concentration effects (2,4-Dichlorophenoxyacetic acid, or 2,4-D), and differential genotype responses, is shown for *in vivo* explants from four genotypes in Fig. 5*A*. The high sensitivity to 2,4-D for callus growth, compared to that of 1-Naphthaleneacetic acid (NAA), is shown in Fig. 6 for *in vitro* explants.

When studied using *in vitro* derived explants on two types of CIM basal media, at the concentrations tested, 2,4-D had a far greater effect than did NAA; however, NAA had a stronger effect when it came to the proportion of explants with shoot and root number from leaf explants (Figures S1-S6 E5ai and E5aii). Differential effects of auxin sources, especially when using 2,4-D, have also been found in other species. For example, for *in vitro* androgenesis in rice 2,4-D resulted in greater callus induction and also greater induction of green (vs. albino) shoots from induced calluses compared to that using NAA (Mishra and Rao 2016). In *Agrobacterium*-mediated genetic transformation of the carnivorous plant *Nepenthes mirabilis,* concentration of 2,4-D was important but not that for NAA (Miguel *et al*. 2020).

On CIM19 basal media, the rate of callus growth from the different explant types were highly correlated using data from the various hormone treatments, with much lower and sometimes negative correlations between explants for root and shoot production traits (Figures S7-12 E5ai). Overall, on CIM23 basal media comparing explant types and hormone

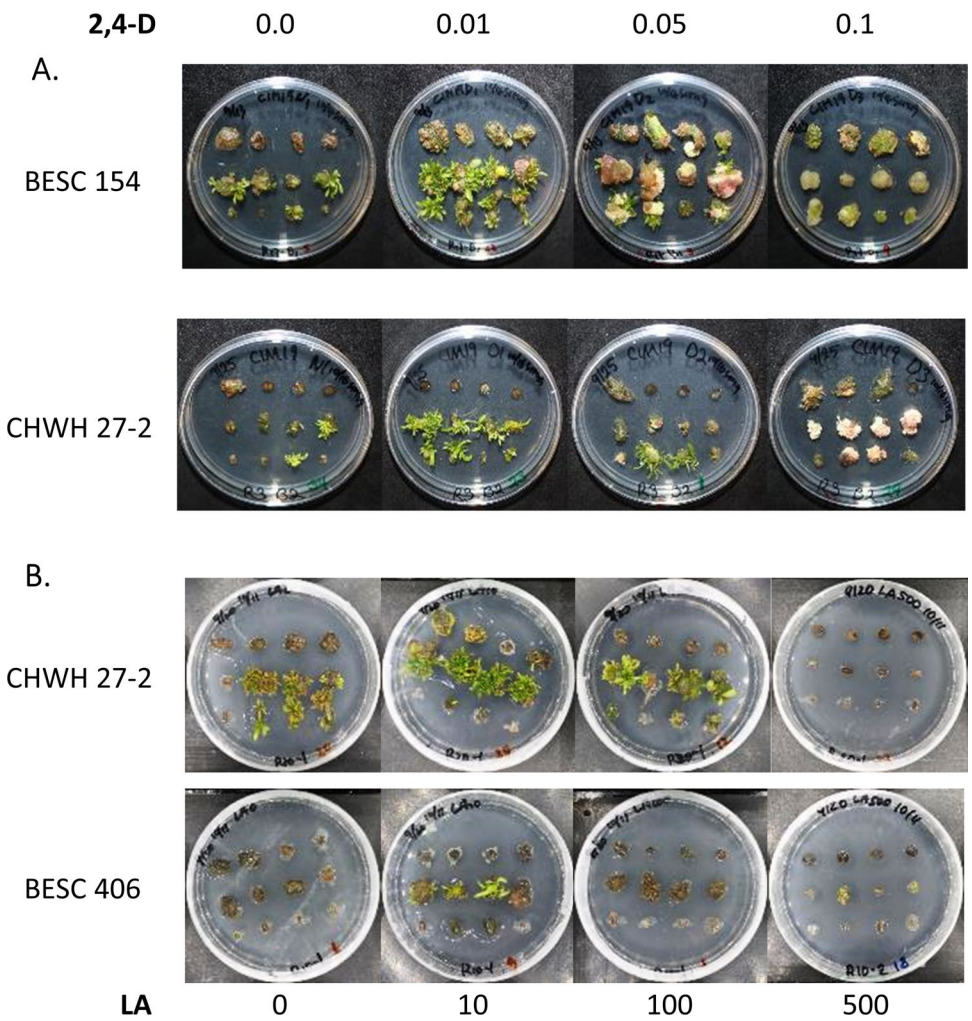

Figure 5. Effect of varying auxin and lipoic acid concentration on indirect *Populus trichocarpa* Torr. and Gray shoot regeneration from *in vivo* explants in two pairs of genotypes. Data shown in relation to (*A*) 2,4-dichlorophenoxyacetic acid (2,4-D) concentration in mgL^{-1} from experiment E5 and (*B*) lipoic acid concentration in µM from experiment E9, each for two *P. trichocarpa* wild genotypes. For (*A*), the explants were placed on CIM19 for 21 d with varying levels of 2,4-D, then transferred to SIM19 with thidiazuron (TDZ) at 0.132 mg L^{-1} for 42 d. For (*B*), explants were placed on CIM19 containing different levels of LA, then SIM19 containing different levels of LA. The three *horizontal rows* of explants in each Petri dish, from *top* to *bottom*, are leaf discs, stems, and petioles.

treatments, shoot number and callus size had a negative correlation (Figure S13 E5aii), as did the proportion of explants with shoots versus callus (Figure S14 E5aii). Shoot and root numbers were only weakly positively correlated (Figure S16 E5aii), while proportions of explants with shoot versus root were positively correlated (Figure S16 Eaii).

For *in vivo* derived explants, both NAA and 2,4-D concentrations has substantial effects on measures of callus, root, and shoot growth, with the relative effects varying among explant types. Stem had the highest overall rate of callus regeneration, leaves gave the highest rate of root regeneration at higher levels of NAA, and stems followed by petioles had much higher rates of shoot regeneration compared to leaf explants (Figures S1-S6 E5b). Correlations among explant types for most traits were generally strong and positive, especially for stem and petiole with respect to shoot regeneration and shoot number. Pooled over the explant types and treatments, correlations among traits were weak or negative, with the exception of proportion of explants with shoots versus proportion of explants with callus; thus, more callus growth appeared to lead to more potential for shoot growth. However, the correlation of shoot number with callus size was much weaker, suggesting that too much callus proliferation may reduce capacity for shoot differentiation (Figures S7-S16 E5b).

E6: Auxin and Cytokinin Combinations In this experiment combinations of the auxins 2,4-D and NAA with the cytokinins benyzyladenine (BAP), 2-isopentenyladenine (2iP), and kinetin in BM19 medium (tested in E3) were tested on *in vivo* explants harvested from 16 genotypes. Not surprisingly, treatments and explants were statistically significant sources of variance for all studied traits, as was treatment x explant interactions, although there was a smaller overall effect. For DB1-DB6 with variations of 2,4-D and BAP (DB media designations are given in Table S3), stems were the most regenerable tissue with respect to callus and shoot production over all combinations studied, roots were never observed, but petioles had only slightly lower rates of shoot regeneration than did stems (Figure S1-S6 E6a). Petioles and stem, callus and shoot regeneration responses were strongly correlated, much more so that other explant combinations

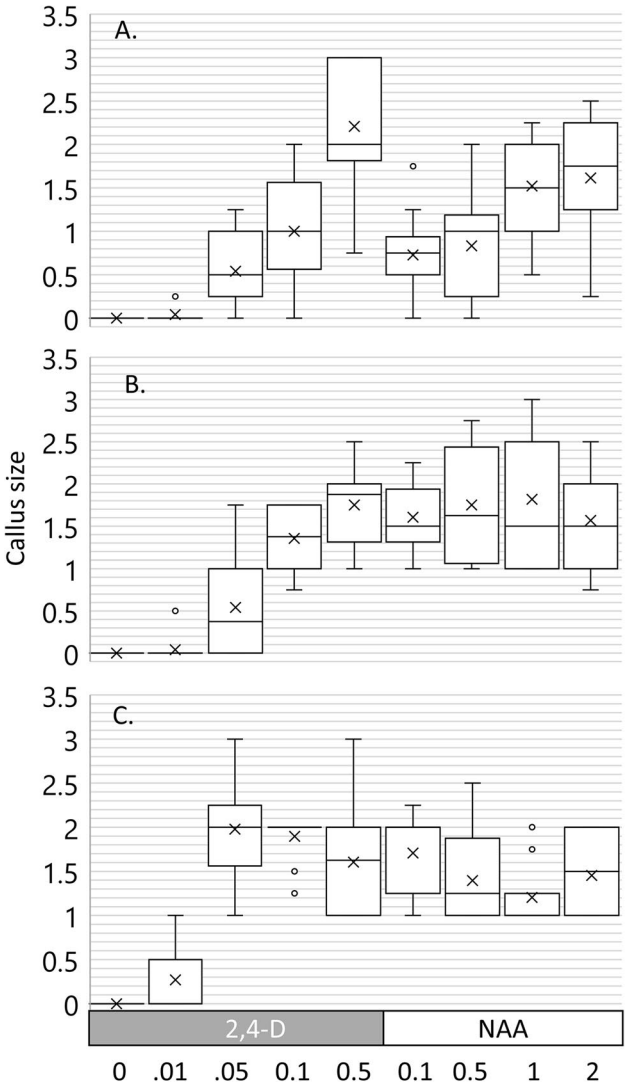

Figure 6. Effects of auxin concentration on generation of *Populus trichocarpa* Torr. and Gray callus. *Box plots* show callus size averaged over genotype using CIM19 in experiment E5 (*in vitro* explants), in relation to varying concentrations in mgL^{-1} of 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) for (*A*) leaf, (*B*) petiole, and (*C*) stem explants. "x" marks show the mean, and *horizontal lines* the median. The *boxes* themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

(Figure S7-S10 E6a). Considered over all explants and treatments, shoot number and callus size were highly correlated, mainly a result of both being more elevated in stems compared to other explant types (Figure S11 E6a).

Treatments DP1-DP6, where 2,4-D and 2iP were varied, showed a very similar pattern, though leaves had higher regeneration rates and even gave rise to a low rate of root regeneration; stems, though generally superior to petioles for callus regeneration, were similar to petioles in shoot regeneration rate (Figure S1-S6 E6b). Correlations among the different explant types were similar, and correlations between shoot and callus regeneration, or between shoot and root regeneration, considered over all explant types, was weak to negative (Figures S13-S14 E6b). Treatments DK1-DK6, where 2,4-D and kinetin were varied, showed very similar patterns (Figures S13-S14 E6c).

For treatments involving NAA as the auxin, callus regeneration was markedly higher for stems vs. other explants, though rates of shoot regeneration were similar (Figures S1-S6 E6d, e, and f). The same was true for NAA combinations with BAP and kinetin, which caused a strong correlation between callus and shoot regeneration when analyzed over all explant types (Figure S1-S6, S9, E6e and E6f).

Overall, the combination of 2,4-D 0.01 mg L^{-1} and BAP 0.5 mg L^{-1} produced the highest rate of shoot regeneration (Fig. 5*A*) and the highest heritability (DB1 in Table S2, Ma *et al*. 2022) from stem explants. This hormone treatment was therefore selected for our GWAS regeneration and transformation studies.

E7: Melatonin and Serotonin Combinations In E7, the effects of varying concentrations of melatonin and serotonin were studied using *in vivo* derived explants from 19 genotypes. Treatment and treatment x explant effects were statistically significant for all traits (Table S1, Ma *et al*. 2022). It was found that in none of the concentrations studied did either compound, singly or together, improve the rate of callus or shoot regeneration (Figures S1-S6 E7). For proportion of explants with shoots, there was a steep decline with increasing concentrations of either melatonin or serotonin (Figure S4 E7). This was also observed, though less steep, with proportion of explants with callus (Fig. 7 and S1 E7). In some studies, these hormones can act as auxins or cytokinins (Murch *et al*. 2001; Erland *et al*. 2015) and can be affected by light and temperature. Therefore, their effects in plant tissue culture vary greatly (Erland *et al*. 2016), and their physiological effects are poorly understood (Byeon *et al*. 2016; Sánchez-Barceló *et al*. 2016; Wei *et al*. 2017). These results appear to be the first reports of melatonin and serotonin effects on poplar regeneration.

E8: Antibiotic Treatments In E8, Plant Preservative Mixture (PPM) and benomyl were tested on 8 genotypes derived from *in vivo* explants, under two types of growth chambers (LED and fluorescent lighting) to test their ability to control contamination (because *in vivo* explants were employed for GWAS, contamination was a continued challenge). Effects on callus growth were small and not statistically significant, though effects on shoot and root regeneration were significant (Table S1, Ma *et al*. 2022). Responses seemed to be similar in the two types of growth

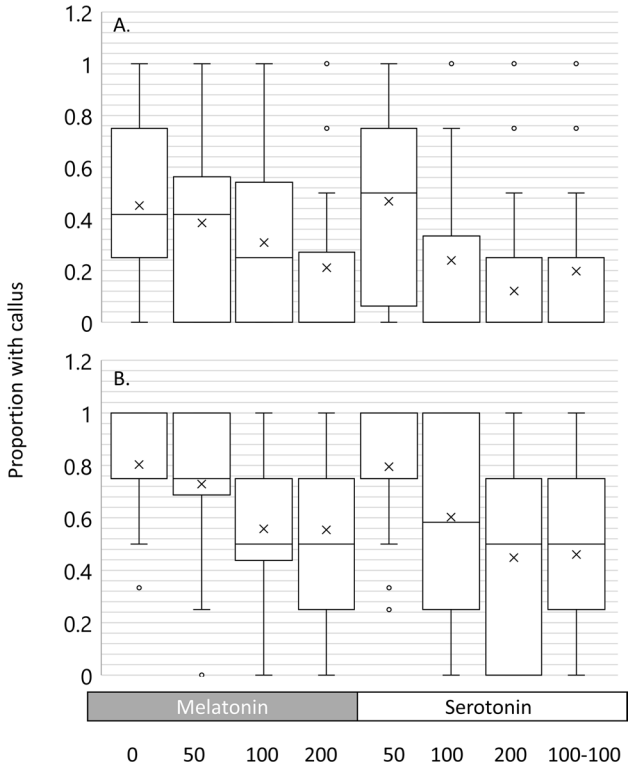

Figure 7. Melatonin and serotonin effects on generation of *Populus trichocarpa* Torr. and Gray callus. *Box plots* of proportion of explants with callus in relation to varying concentrations in µM of melatonin and serotonin using (*A*) petiole and (*B*) stem *in vivo* explants from experiment E7. "x" marks show the mean, and *horizontal lines* the median. The boxes themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

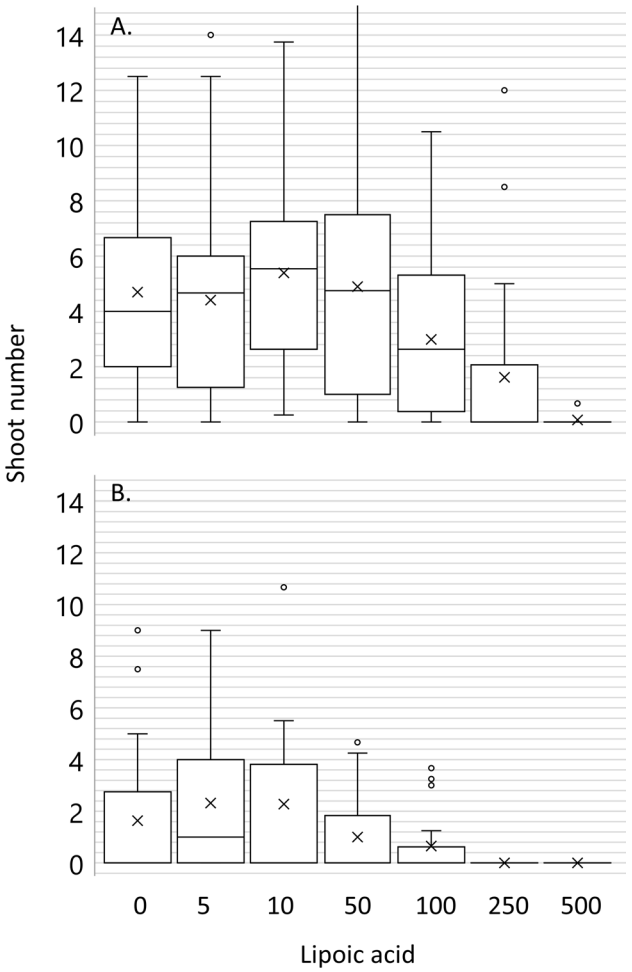

Figure 8. Effect of lipoic acid on *Populus trichocarpa* Torr. and Gray shoot regeneration. *Box plots* of shoot number per explant in relation to varying concentrations in µM of lipoic acid are shown for (*A*) stem and (*B*) petiole *in vivo* explants from experiment E9. "x" marks show the mean, and *horizontal lines* the median. The boxes themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

chambers, and it was found that there was only a modest though slightly negative effect on regeneration responses at the highest antibiotic concentrations (Figure S1-S6 E8a, S1-S6 E8b). The much higher rate of stem regeneration gave rise to correlations between proportion of explants with shoot vs. root, and proportion of explants with shoot vs. callus (Figures S7-S8 E8a, S7-S8 E8b). Both compounds are broad spectrum fungicides commonly used to control contamination in tissue culture (PPM is also a general biocide). It has been shown, however, that many endophytes persist in tissue culture even with the use of PPM (Thomas *et al*. 2017), and that it may negatively affect regeneration in certain species (Compton and Koch 2001). Furthermore, benomyl and PPM can cause phytotoxicity at high concentrations (Paul *et al*. 2001). At the rates that were studied, it was only the highest where negative effects on regeneration were observed.

E9: Lipoic Acid In E9, lipoic acid (LA) was tested on *in vivo* derived plants from 16 genotypes. Treatment effects were large and statistically significant except for roots (Table S1, Ma *et al*. 2022), which only rarely regenerated, and only from stems. The highly deleterious effects of high LA concentrations for shoot regeneration are clear in Figs. 5*B* and 8. Its effects also tend to be similar in regard to the other parameters studied (response percentages and regeneration numbers) (Figures S12-S15 E9). Although there is a weak tendency for regeneration to improve from zero (control) to 10 µM, the major effect of LA is clearly deleterious for both generation of callus and shoot (Figures S1-S6 E9). The biosynthetic pathway and physiological mechanism for LA is unclear; however,

it is commonly used to reduce browning in tissue culture and protect against oxidative stress, and thus may improve transformation efficiency (Xiao *et al*. 2018). Such effects have been observed in soybean, tomato, cotton, and wheat (Dan *et al*. 2009), and in Mexican lime (Dutt *et al*. 2011). This appears to be the first report of LA effects on regeneration in poplar.

E10: Activated Charcoal and Ascorbic Acid In E10, the effects of activated charcoal (AC) or ascorbic acid (VC) were studied on *in vivo* derived explants from 16 genotypes. The effects of these treatments were statistically significant for all traits, though had positive effects only on shoot and callus traits (Table S1 in Ma *et al*. 2022). Although it had little effect on callus, activated charcoal reduced the rate of shoot regeneration considerably (Fig. 9), driving it to zero at 0.5 and 1.5 g L^{-1} (Figures S1-S6 E10a). Correlations among explant types for shoot regeneration was strongest when stems and petioles were considered (Figures S7-S8 E10a). VC has no appreciable negative or positive effects on regeneration (Figures S1-S6 E10b). VC is widely believed to function as an antioxidant (Mridula *et al*. 2017; Lieber 2019; Khajuria *et al*. 2020; Mishra *et al*. 2020) but has also been known to be cytotoxic (Clément *et al*. 2001). AC is often used as an additive which can reduce oxidative stress, absorb inhibitory compounds and toxic metabolites, thus promoting plant development (Thomas 2008). AC is also often used in tissue culture to improve cell growth and development during plant propagation (Garvita and Sahromi 2019), as well as seed germination, seedling development (Kim *et al*. 2019), root formation (Mao *et al*. 2018), somatic embryogenesis, and plant regeneration (Mittal *et al*. 2016).

E11: Silver Nitrate In E11, silver nitrate ($AgNO_3$ or SN) was tested on *in vivo* derived explants from 16 genotypes. The main effects for SN treatment were statistically significant only for shoot traits (Table S1 in Ma *et al*. 2022). There were no clear positive effects of SN at any concentration, and high concentrations strongly inhibited shoot regeneration from stems (Fig. 10, S1-S6 E11), an effect that was even more dramatic with petiole explants (Figure S2 and S6 E11). Correlations were moderate and positive among shoot, root, and callus number and proportion, respectively (Figures S11-S14 E11). Silver nitrate has been identified as a potent ethylene regulator that can control browning in explants (Kumar *et al*. 2009), and is important to calcium and polyamine signaling. This appears to be the first report of its effects on regeneration in poplar (Mahendran *et al*. 2019).

E12: Light Intensity and Quality In E12, the effects of variable light spectra and intensity from LED lights were tested with *in vivo* derived explants from 4 genotypes. White light and far red light sources were used, and the intensity and mixture varied. The effects of the light treatments and treatment x explant interactions

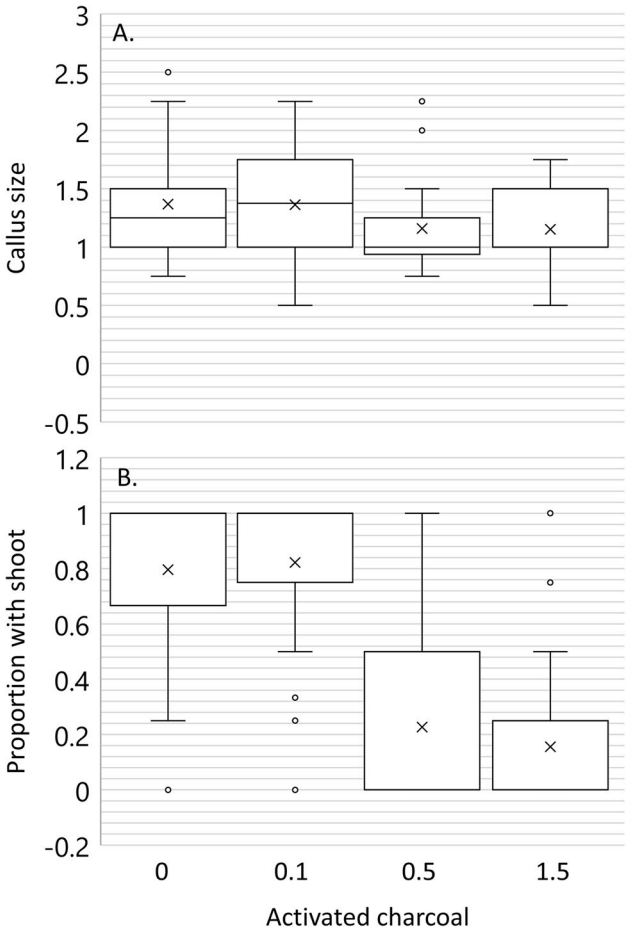

Figure 9. Effect of activated charcoal (gL^{-1}) on *Populus trichocarpa* Torr. and Gray shoot regeneration in *in vivo* stem explants. *Box plots* of (*A*) callus size, averaged over genotypes and (*B*) proportion of explants with shoots from experiment 10. "x" marks show the mean, and *horizontal lines* the median. The boxes themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

were generally small and not statistically significant (Table S1 in Ma *et al*. 2022). With the exception of root regeneration, increased white light intensity in the absence of far red light tended to improve regeneration for most explant types, and this increase was marked for shoot regeneration from stem explants (Figures S1-S6 E12). Similar results were found in studies of *Populus berolinensis* root explants, where the highest regeneration was observed under fluorescent lamps (Pavlichenko *et al*. 2020). In contrast, when far-red light was present, the effects of increased white light intensity was negligible and inconsistent, though the shoots elongated and grew larger under far red light (data not shown). With the advent of LED lights, consistent intensity and quality of light is easier to obtain and energy costs are lower, thus it has become popular in plant tissue culture. Light sources are known to be important

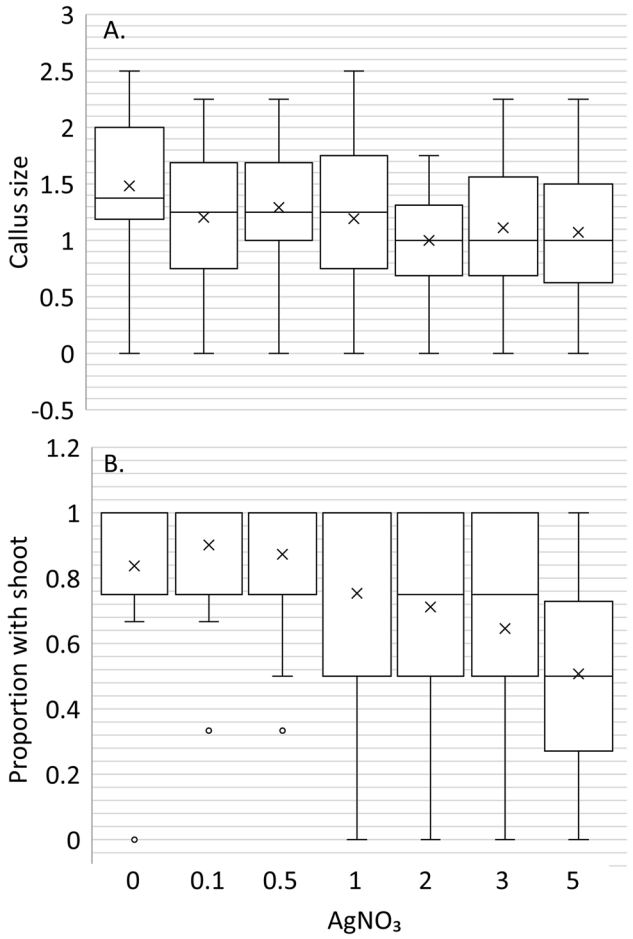

Figure 10. Silver nitrate (mgL^{-1}) effects on *in vivo Populus trichocarpa* Torr. and Gray stem explants for generation of callus and shoots. *Box plots* shown of (*A*) callus size and (*B*) proportion of explants with shoots. Data were averaged over genotypes in relation to silver nitrate concentration in experiment E11. "x" marks show the mean, and *horizontal lines* the median. The boxes themselves represent the interquartile range (IQR) of the data. The whiskers of the boxplot represent the minimum and maximum of the data (barring outliers). Any data 1.5 times greater or less than the third quartile and first quartile, respectively, is considered an outlier and is represented with *small circles*.

to control vitrification rates (Muneer *et al*. 2018), shoot and root tissue production, chlorophyll content (Poudel *et al*. 2008; Hung *et al*. 2016; Ramírez-Mosqueda *et al*. 2017), shoot quality, and regeneration. Many of these effects were also reported in poplar (*Populus euramericana*: Kwon *et al*. 2015).

Conclusions

In this study, a very large number of factors that can affect callus and shoot regeneration were examined. Many of these factors have never before been studied in poplar, and employed a large sample of diverse genotypes to give a general picture of what works and what does not in *P. trichocarpa*. Included were explant types and sources; direct versus indirect regeneration methods; basal medium salts and micronutrients; auxin and cytokinin types, concentrations, and combinations; oxidant and ethylene mitigation treatments; antibiotics for contamination control; light quality and intensity; sugar concentration; and the effects of the new (for poplar) growth modifying chemicals melatonin and serotonin.

As a result of this wide exploration, treatment combinations that substantially elevated regeneration in diverse genotypes were identified. Moreover, in a companion paper (Ma *et al*. 2022), treatments with high heritabilities suitable for GWAS of *in vitro* regeneration and transformation were also identified. Though by no means fully optimized, the best regeneration procedure we identified used an indirect regeneration system, *in vivo* stem explants in MS media modified with reduced ½ KNO_3 and Meso (BM19), 30% sucrose, a combination of 2,4-D 0.01 mg L^{-1} and BAP 0.5 mg L^{-1} for callus induction, then 0.132 mg L^{-1} TDZ for shoot induction, and 5 μM lipoic acid in both CIM and SIM.

Appendix (list and links to electronic supplementary materials)

Supplemental figures and tables referenced in the text include: Figures S1-S4 E1; S1-S25 E2; S1-S16 E3a; S1-S6, S16 E3b; S1-S11 E4; S1-S12 E5ai; S1-S6, S13-S14, S16 E5aii; S1-S16 E5b; S1-S11 E6a; S1-S6, S13-S14 E6b; S13-S14 E6c; S1-S6 E6d; S1-S6, S9 E6e; S1-S6, S9 E6f; S1-S6 E7; S1-S8 E8a; S1-S8 E8b; S1-S6, S12-S15 E9; S1-S8 E10a; S1-S6 E10b; S1-S6, S11-S14 E11; S1-S6 E12; Tables S1-S3.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11627-022-10301-9.

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Authors contribution Ma and Strauss designed the experiments; Ma and Peremyslova oversaw and executed the experiments and data collection; Nagle, Duan, and Jiang oversaw and executed the data analysis and graphics; Goddard formatted the graphics and tables; Strauss, Ma and Goddard wrote the manuscript; all authors edited and approved the manuscript.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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