Toward Optimization of *in vitro* Regeneration and Transformation in Wild Black Cottonwood *(Populus trichocarpa)*

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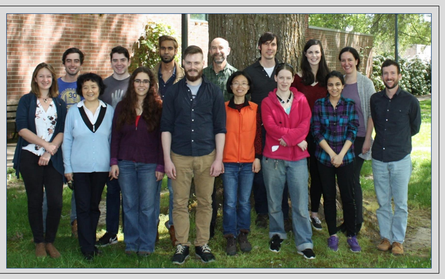


Summary

As part of a Genome Wide Association Study (GWAS) to identify genes that modify the amenability of poplars (*Populus*) to *in vivo* and *in vitro* regeneration and transformation, we studied a wide variety of media components, hormones, and transformation procedures in a sample of resequenced wild cottonwood genotypes.

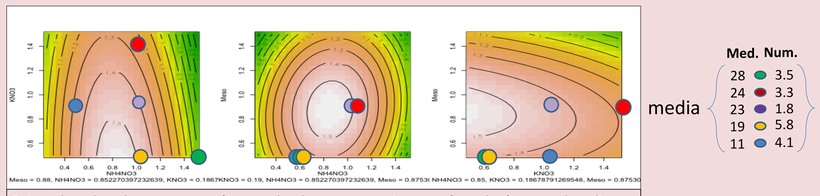
We analyzed the effects of 36 permutations of MS basal media [factorial combinations of NH4NO3, KNO3, and MESO (CaCl2, MgSO4 and KH2PO4) levels], resulting in the identification of five media that gave consistent and high rates of organogenesis. When variable levels of sucrose (1.5, 2.0, 2.5, and 3.0%) in the selected MS media were studied, the highest sucrose level supported the strongest callus and shoot regeneration from all genotypes.

We investigated several factors that affect *Agrobacterium*-mediated transformation in three genotypes using the 2X-35S promotor driving an enhanced GFP gene (in Pc2300 backbone). Acetosyringone (AS) increased transient and stable transformation. The rate of recovery of transgenic plants was higher when geneticin was the selection agent instead of kanamycin.

The relationship of transformation rate to duration (0 to 21 days) of time on callus induction medium (CIM) with geneticin (after co-cultivation) was examined, and the optimal durations varied widely among genotypes. The best transformation rate from the genome sequenced clone *P. trichocarpa* Nisqally-1 was 6.3%, and occurred when there was only two days of co-cultivation on CIM (with AS) before explants were moved to shoot induction medium (SIM). Direct regeneration (no CIM phase) gave a high rate of shoot regeneration from many clones but almost all regenerants were escapes. Customizing the CIM:SIM period for individual genotypes seems to be an important step in transformation.

Medium 19 gave highest shoot regeneration from leaf & stem explants

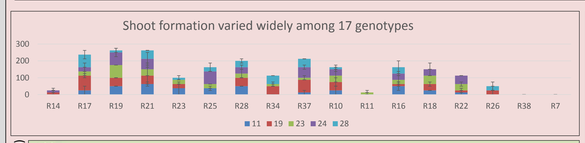
Average # of shoots per leaf explant from *in vitro* materials

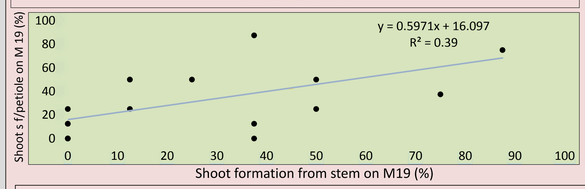


media

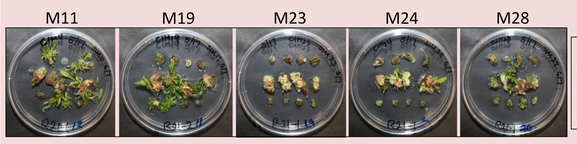
Med.	Num.
28	3.5
24	3.3
23	1.8
19	5.8
11	4.1

Points plotted on the response surface are the shoot regeneration rates from the five tested media. Results suggest that media could be further optimized.





Rate of shoot regeneration on five types of basal media using greenhouse materials as sources of explants. Stem explants above, and stem vs. petiole explants below on plates. Stem and petiole regeneration on M19 (shown below) was stronger than on other media, and was correlated among the explant types (bottom graph).



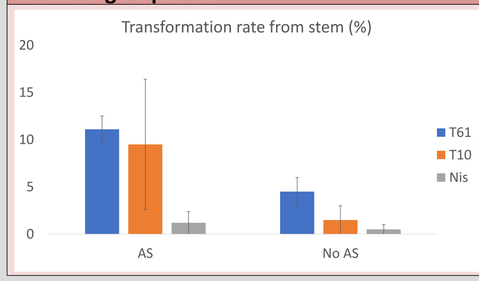
Example of the wide variation in shoot formation from genotype R21 among the 5 tested basal media.

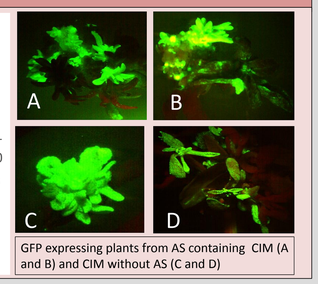
Transformation protocol development for *Populus trichocarpa*

- Three genotypes were randomly selected for study
- Plants were grown in WPM hormone-free medium (example of source plants shown to right)
- Leaf and stem (including petiole) explants were co-cultivated with 2x35S::eGFP in Pc2300 (contains NPTII selection)
- Two to four plates per genotype and 20-30 explants per plate
- Stable GFP expression verified under GFP microscope
- Three factors studied were:
- AS vs. no AS in co-cultivation CIM
- Geneticin (G418) vs. kanamycin selection
- Duration on CIM with G418 selection

In vitro plants growing in WPM

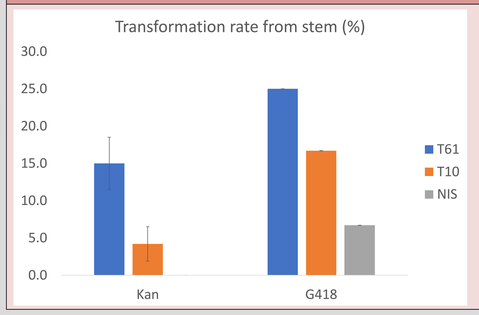
Transformation efficiency doubled after 2 days of co-cultivation on CIM containing 50 µM AS

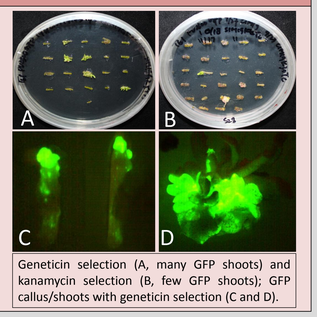




GFP expressing plants from AS containing CIM (A and B) and CIM without AS (C and D)

Geneticin (5 mg/L) selection increased transformation rates over that from kanamycin selection for three genotypes tested





Geneticin selection (A, many GFP shoots) and kanamycin selection (B, few GFP shoots); GFP callus/shoots with geneticin selection (C and D).

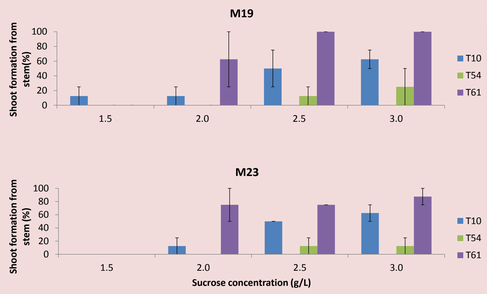
Basal medium screening for shoot regeneration

- 36 basal media were screened (0.25X, 0.5X, 1X, 1.5X of MS in NH4NO3, KNO3, Mesos)
- Leaf, stem and petiole explants were tested
- 12 explants per plate, 2 plates per genotype, studied
- Explants were cultured on callus induction medium (CIM) for 20d in dark and the basal medium was supplemented with 2µM 2iP and 10µM NAA
- Explants were transferred onto shoot induction medium (SIM) for 42 days under light and supplemented with 0.6µM TDZ

Table 1. Part of Optimization Table (X is the amount relative to MS)

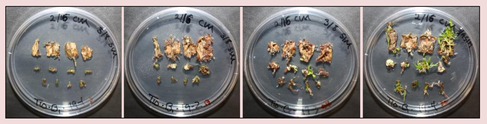
Treat	NH4NO3	KNO3	Meso: CaCl2, MgSO4, KH2PO4	Micronut trient
1	0.25X	0.5X	0.5X	1X
2	0.25X	1X	0.5X	1X
3	0.25X	1.5X	0.5X	1X
4	0.25X	0.5X	1X	1X
5	0.25X	1X	1X	1X
6	0.25X	1.5X	1X	1X
7	0.25X	0.5X	1.5X	1X
8	0.25X	1X	1.5X	1X
9	0.25X	1.5X	1.5X	1X
10	0.5X	0.5X	0.5X	1X
11	0.5X	1X	0.5X	1X
12	0.5X	1.5X	0.5X	1X
13	0.5X	0.5X	1X	1X
14	0.5X	1X	1X	1X
15	0.5X	1.5X	1X	1X
16	0.5X	0.5X	1.5X	1X
17	0.5X	1X	1.5X	1X
18	0.5X	1.5X	1.5X	1X

High concentrations of sucrose enhanced shoot formation from *in vitro* stems



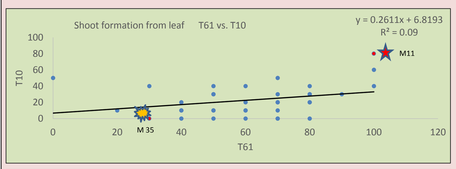
Two basal media were studied (19 and 23) and three wild genotypes (T10, T54, and T61).

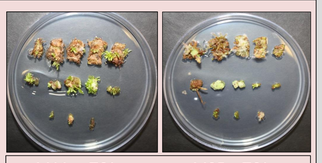
Though regeneration rates varied widely among genotypes, the rate was maximized at the higher sucrose concentrations tested (2.5-3.0 %).



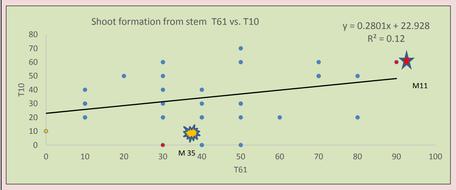
Left) are images of variation in shoot regeneration with different concentrations of sucrose for genotype T10

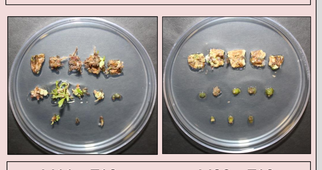
We identified media that supported high shoot regeneration from diverse clones and explant types





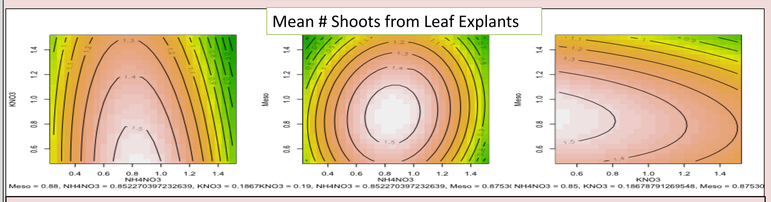
M11: T61 M35: T61





M11: T10 M30: T10

M11 (low salt) gave the best regeneration from both leaf and stem in the two clones, whereas M35 and M30 (high salt) gave poor responses in both.



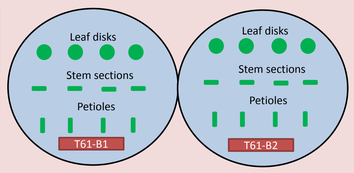
Response surface analysis for shoot regeneration showed an optimum was observed for Mesos vs. NH4NO3 (center); an optimum was achieved beyond the limit of the experimental levels for others (left and right).

We studied genetic variation in shoot regeneration in five basal media

Five basal media that gave good regeneration were tested in an indirect regeneration system (Table 2). One experiment had leaf, stem and petiole explants derived from 6 genotypes of *in vitro grown* plants, and a second expt. had the same explant types from 17 genotypes of *in vivo* grown plants.

Table 2. Nutrient components of tested media.

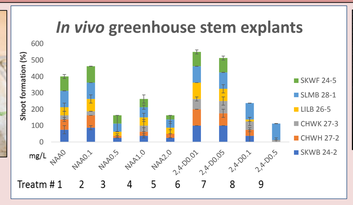
Treat-ment	NH4NO3	KNO3	Mesos: CaCl2, MgSO4, KH2PO4	Micro-nutrients	Fe-EDTA
11	0.5X	1X	0.5X	1X	1X
19	1X	0.5X	0.5X	1X	1X
23	1X	1X	1X	1X	1X
24	1X	1.5X	1X	1X	1X
28	1.5X	0.5X	0.5X	1X	1X



Explant layout on Petri dishes

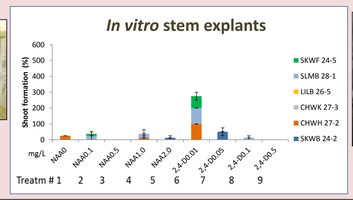
Explants from *in vivo* greenhouse plants had superior shoot formation to explants from *in vitro* plants

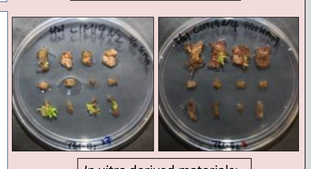
In vivo plants



In vivo derived materials: Auxin treatments 2 & 4

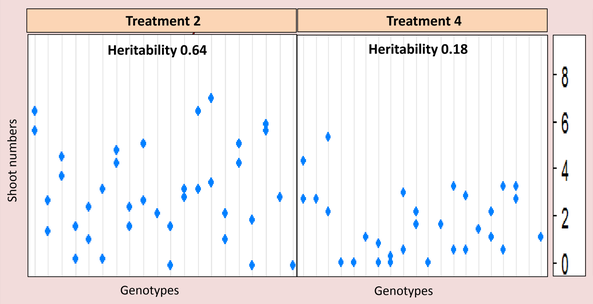
In vitro plants





In vitro derived materials: Auxin treatments 2 & 4

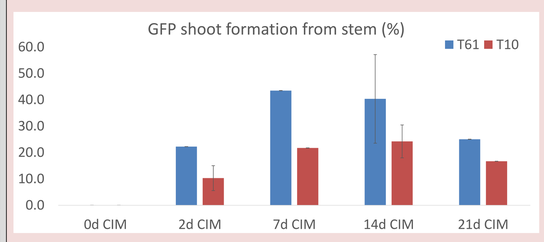
Broad-sense heritability varied widely among different auxin treatments, and is a useful parameter for helping to select optimal treatments for use in GWAS studies



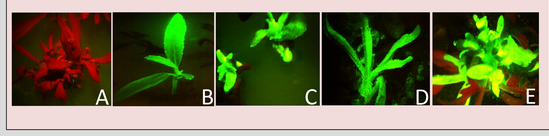
Variation among genotypes and replicate blocks (pairs of points per vertical line) from a study of heritability in shoot regeneration.

Stem explants of 20 wild genotypes from greenhouse grown plants were studied. Treatment 2 gave high heritability (left) while treatment 4 (right) showed low heritability.

Transformation efficiency was doubled in genotypes T10 and T61 when CIM was optimized at 7 to 14 days for each genotype



Duration of callus induction phase is the key step for successful transformation of *P. tricocarpa*. No CIM or short CIM treatments (2d-3d) gave many shoots, but 80-100% are escapes (data not show); longer CIM treatment (21d) decreased transformation efficiency.



Shoot formation from stem explants on different duration of callus induction medium (from left to right, 0d to 21d, as graphed above

Conclusions

- Shoot regeneration from two tested genotypes of *P. trichocarpa* varied widely among different strengths of macro and micro element-containing media of 64 tested
- Among 5 selected basal media, low KNO_3 and MESO (M19) gave better organogenesis for all genotypes; this basal medium was selected for further optimization treatments
- Explants from greenhouse-grown *in vivo* plants showed markedly higher shoot regeneration than from *in vitro* grown plants
- Shoot regeneration was greatest at a high level of sucrose for all genotypes
- Acetosyringone induction in CIM during co-culativation with *Agrobacterium* greatly improved the rate and intensity of stable GFP from stem explants
- Geneticin was far more efficient for selection and regeneration of transgenic callus and shoot selection than was kanamycin
- 7 days and 14 days on CIM with selection greatly improved transgenic shoot recovery compared to our standard CIM treatment of 21 days
- Auxin concentrations and types affect rate and heritability of shoot regeneration, and are thus important to consider when selecting conditions for GWAS

Acknowledgements

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