Developing a micropropagation system for the commercialization of a hazelnut industry in Ontario

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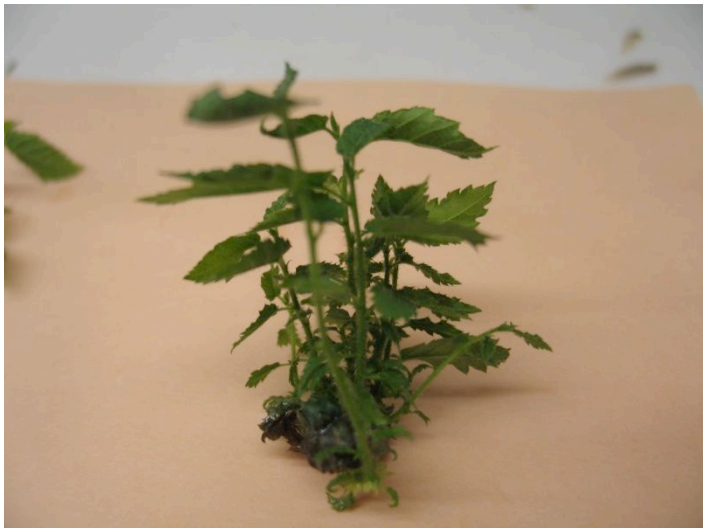
Outline

- Plant Cell Technology Laboratory
- Objectives of research
- Challenges
- Micropropagation stages
- Experiments
  - Initiation, Proliferation, Rooting
- Results and future directions for research

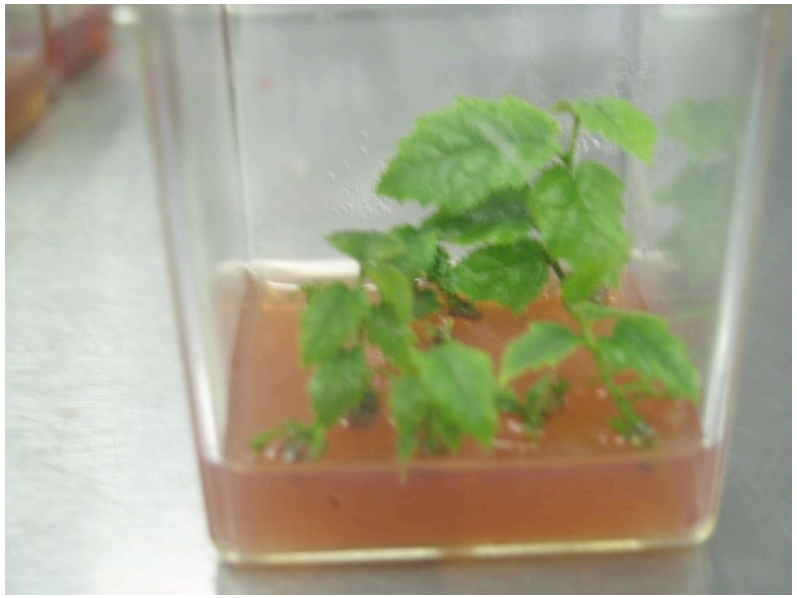
Plant Cell Technology Laboratory – University of Guelph

- The overall objective of our research programs is to develop novel technologies and products for plant based medicines and the conservation of unique plant species while expanding our knowledge of plant growth and development
- Currently working on projects involving hazelnut, American elm, American chestnut, ginseng



Major objective of hazelnut research

- Develop a micropropagation protocol for hazelnut cultivars suitable for production in Ontario that is
  - *Consistent*
  - *Repeatable*
  - *Efficient*
  - *Optimized*



cv. 'Geneva'

Micropropagation

- Mass production of true-to-type plants

Advantages

- Rapid, high volume multiplication
- Year-round production
- Minimal international restrictions
- Conservation

Micropropagation - Stages

- **Stage 0:** Mother plant selection and preparation
- **Stage I:** Establishing an aseptic culture
- **Stage II:** Proliferation of suitable plant outgrowths
- **Stage III:** Rooting and preparation for growth in the natural environment
- **Stage IV:** Acclimatization and transfer to natural environment

Challenges in Hazelnut Micropropagation

Fungal contamination in forced outgrowth of hybrid cv. 'Slate'

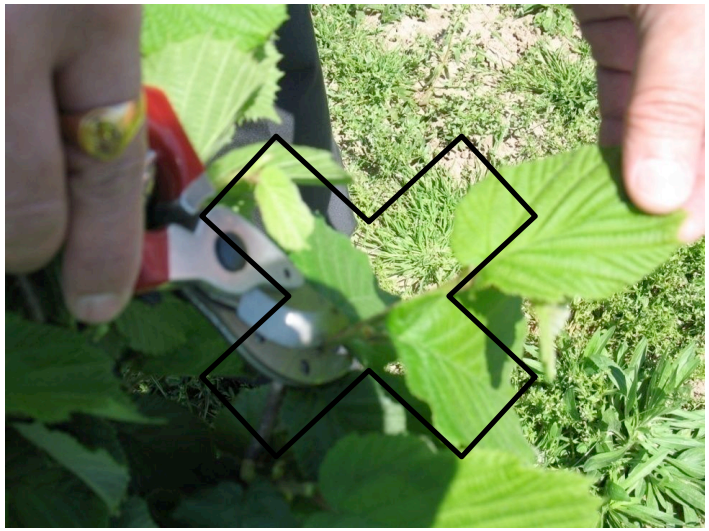
- Hazelnuts are extremely difficult to micropropagate
- Recalcitrant plant
  - Low initiation and multiplication rates
- Microbial contamination, especially in mature plant material
  - Indexing for fungal and bacterial contamination
- Several subcultures required before maximization of desired attributes
- Acclimatization after rooting can be problematic

Challenges specific to Ontario hazelnut project

- Stock plant availability and quality
- Contamination
  - Reduced rates when cleaner (i.e. greenhouse), juvenile material used
- Achievement of high initiation and multiplication rates
- Rooting *in vitro* and *ex vitro*
- Developing sufficient germplasm for rooting and field trials

Stage 0: Sourcing stock plant material

- Best source of material for *in vitro* propagation – shoots from greenhouse-grown juvenile stock plants
- Basal sprouts collected early in season may have potential
- Shoots from the canopy, semi-hardwood, and hardwood material difficult to initiate and multiply
- Difficult to eradicate surface fungal and bacterial contamination
- Current source of plant material –Simcoe, Vineland research stations, private growers (Ernie Grimo, Martin Hodgson)



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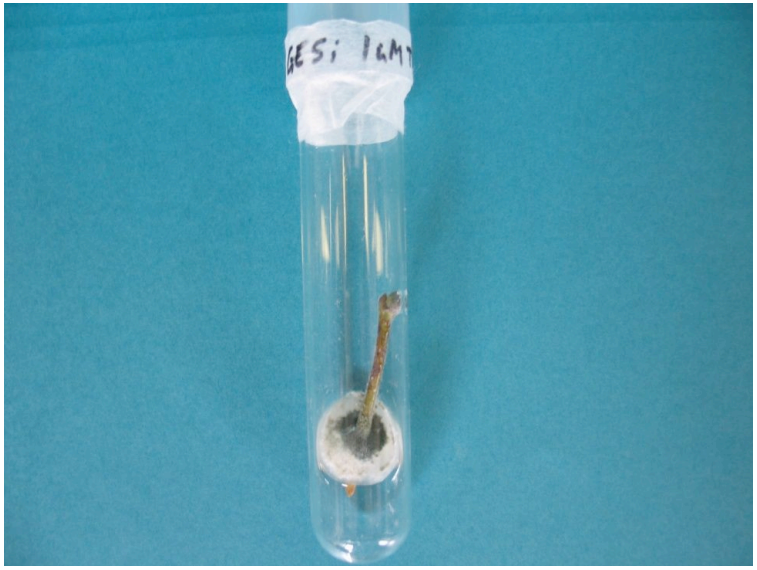
Initial explant surface sterilization procedure

- Soap/water wash – 20 min to 1 hr
- EtOH – 1 min
- dH_2O rinse – 3 min
- 15% soln sodium hypochlorite (NaOCl) + Tween 20 (1 drop) + HCl for pH adjustment
- NaOCl contact time to 30 min. @ 0.2% + 10 min. 20.0% + 10 min. ascorbic acid
- dH_2O rinses -3 x 3 min.
- Anti-fungal pre-treatments thiabendazole and benomyl at 1 $mg \cdot L^{-1}$
- Plant Preservative Mixture (PPM) in DKW media

Survival rates of *C. avellana* L. x *C. americana* M. cv. 'Geneva': Stock plant selection & media

Init Date	Cultivar	Location	Media	Disinfection Proc	No.	Contam & Disc	% Survive	Fungal	% Fungal	Phen	% Phen
06/07/2010	Geneva	GRGH	AC	STD	19	15	21.1	1	6.7	8	53.3
06/07/2010	Geneva	GRGH	CPA	STD	26	18	30.8	4	22.2	7	38.9
21/07/2010	Geneva	VL	CPA10L	STD	11	11	0.0	6	54.5	5	45.5
22/07/2010	Geneva	VL	CPA10Laa	STD	11	11	0.0	6	54.5	5	45.5
06/07/2010	Geneva	GRGH	DKW 3	STD	39	19	51.3	3	15.8	7	36.8
21/07/2010	Geneva	VL	DKW 3L	STD	50	50	0.0	9	18.0	12	24.0
06/07/2010	Geneva	GRGH	DKW 5	STD	63	42	33.3	3	7.1	23	54.8
21/07/2010	Geneva	VL	DKW 5L	STD	11	11	0.0	6	54.5	5	45.5
21/07/2010	Geneva	VL	TIBA1L	STD	36	36	0.0	17	47.2	10	27.8
21/07/2010	Geneva	VL	TIBA5L	STD	28	28	0.0	7	25.0	9	32.1
21/07/2010	Geneva	VL	TIBA10L	STD	26	26	0.0	3	11.5	11	42.3
Total					320	267	16.6	65	24.3	102	38.2



Fungal contamination of field-grown cv. 'Geneva' in liquid DKW media.

Survival rates of *Corylus* genotypes: Stock plant selection & media

Init Date	Cultivar	Location	Number	Contam & Disc	% Survive	Fungal cont	% Fungal	Phen cont	% Phen
17/06/2010	16	HF	47	42	10.6	6	14.3	6	14.3
18/06/2010	28	HF	49	49	0.0	2	4.1	2	4.1
15/06/2010	32	HF	35	35	0.0	6	17.1	5	14.3
07/06/2010	314	HF	24	24	0.0	0	0.0	1	4.2
21/06/2010	409	HF	50	50	0.0	1	2.0	1	2.0
10/06/2010	8-15.5	HF	39	39	0.0	0	0.0	15	38.5
26/07/2010	Gamma	VL	134	134	0.0	113	84.3	0	0.0
22/07/2010	Geneva	GH	147	94	36.1	11	11.7	45	47.9
22/07/2010	Geneva	VL	227	227	0.0	75	33.0	118	52.0
20/07/2010	Halle's Giant	SI	7	7	0.0	5	71.4	0	0.0
19/07/2010	Jefferson	SI	24	24	0.0	21	87.5	0	0.0
26/07/2010	Santiam	VL	48	48	0.0	43	89.6	0	0.0
22/07/2010	Slate	VL	181	181	0.0	83	45.9	51	28.2
09/06/2010	Tondade G	VL	6	6	0.0	0	0.0	2	33.3
Total			1018	960	5.7	366	38.1	246	25.6

Tissue culture growth environment

- Light – concentration of 25 umol m^{-2} s^{-1} cool white fluorescent
- Temperature – 23°C , 29°C (↑ growth rate)
- Photoperiod
  - 16 h day/8 h night

Stage I: Meristem culture for micropropagation of hazelnut

- Long shoots cut into single nodes and planted vertically in medium
- Axillary buds at each node elongate and grow in length
- Layering hybrid cvs. ‘Geneva’ and ‘16’ with 2 or more nodes to reduce apical dominance

cv. ‘Geneva’ 34 days

Culture medium

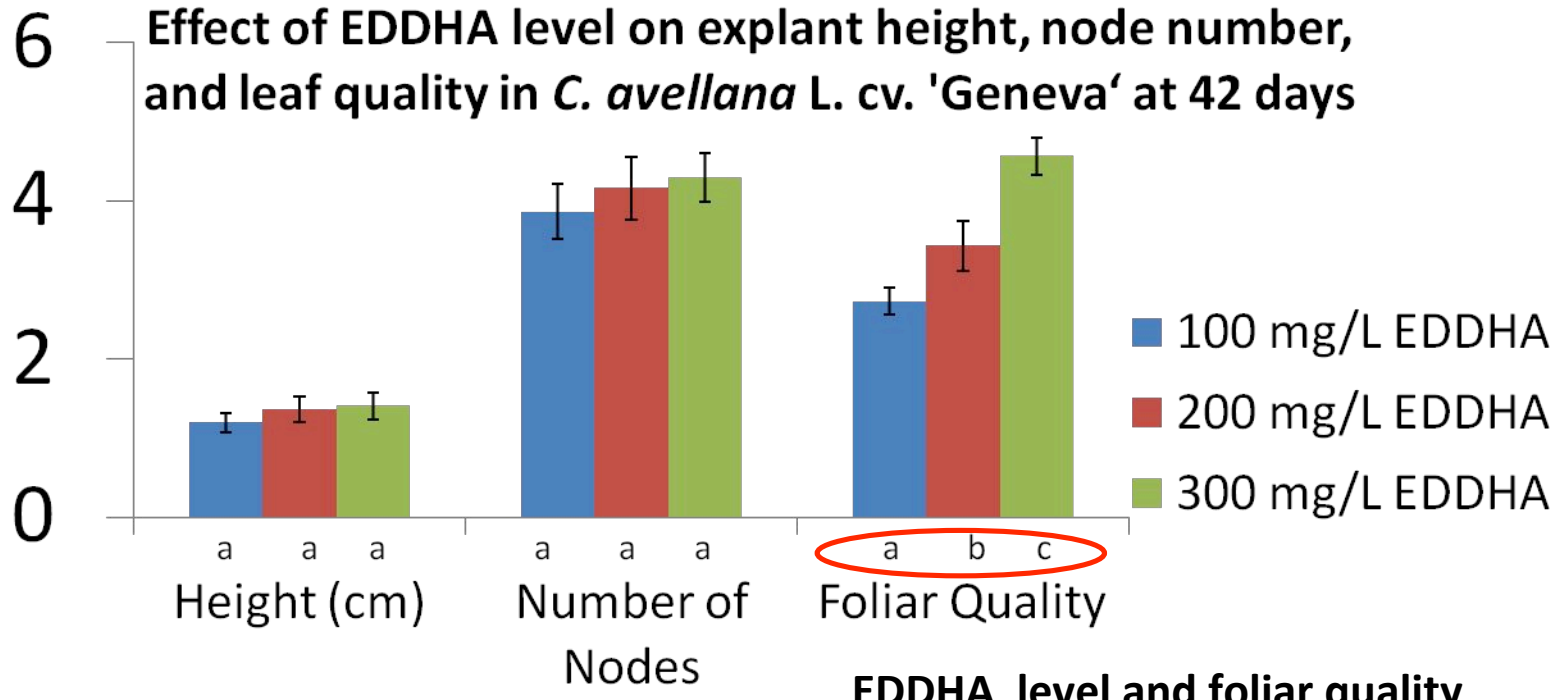
- MS, DKW, WPM, NCGR-COR, and NRM media have been used in hazelnut micropropagation
- For Ontario-grown hazelnut hybrids MS, DKW, and WPM ineffective for shoot initiation and elongation
- Modification of NCGR-COR positive results in both initiation and multiplication
- Several differences between media
  - Fe-EDTA in MS, DKW, WPM
  - Fe-EDDHA in NCGR-COR and NRM

Stage II: Stem elongation and proliferation

Effect of iron source on plant development *in vitro*

- In plants, iron used for chloroplast (precursors of chlorophyll) formation, mitochondria
- Fe deficiency symptoms include leaf chlorosis
- Added in a chelated form to improve plant availability
- Fe-EDTA (ethylenediamenetetraacetic acid) used in MS, DKW, and WPM media
- Photodegrades readily and can lead to Ca^{2+} and Mn^{2+} deficiencies
- EDDHA (ethylenediamene-di-(*o*-hydroxyphenyl acetic acid) used as an alternative
- Does not photodegrade and does not scavenge Ca^{2+} or Mn^{2+}

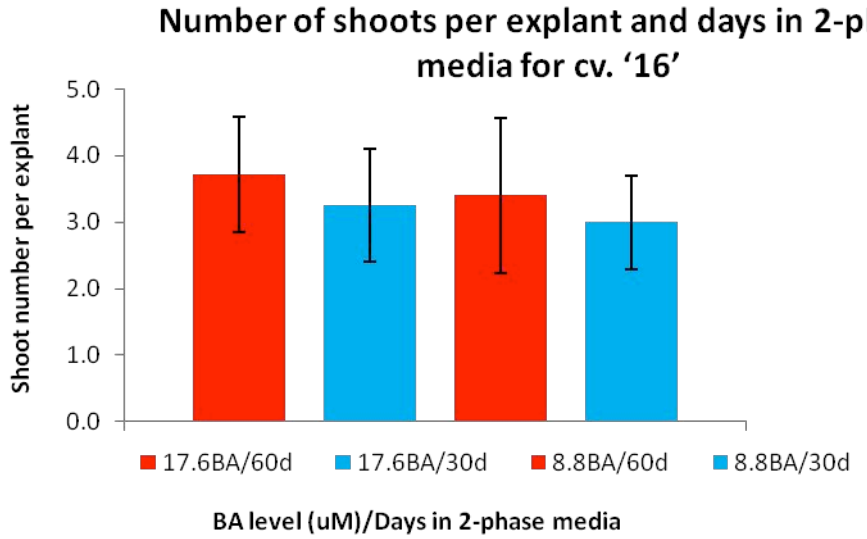
Effect of EDDHA level on explant development in vitro



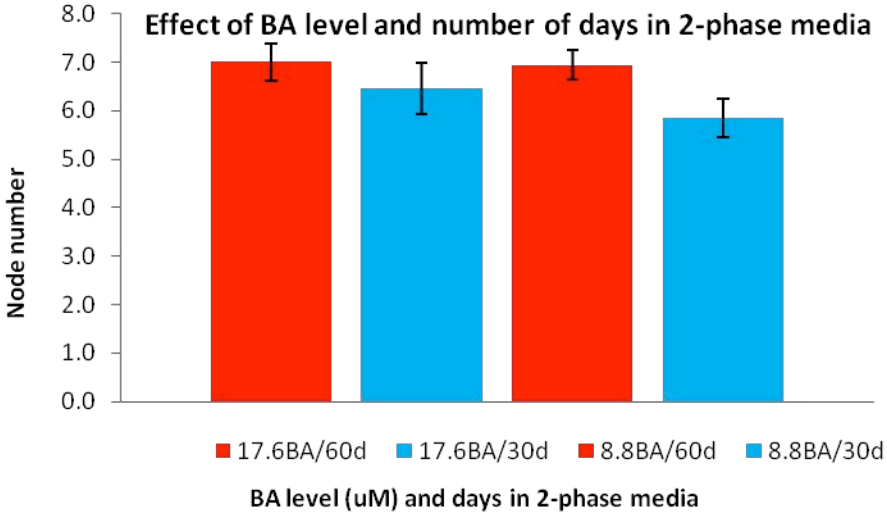
EDDHA level and foliar quality assessment. Foliar Quality 1 = leaves dead; 2 = leaves yellow, large necrotic areas; 3 = moderate leaf necrosis; 4 = no necrosis, light green; 5 = no necrosis, dark green. N = 30.

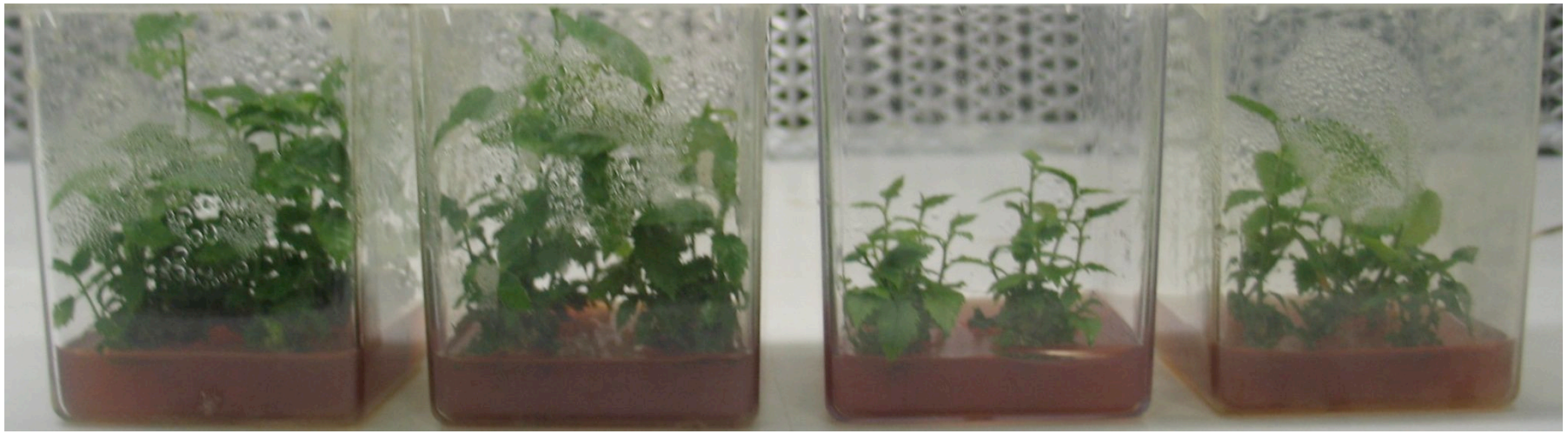
Effect of Double Phase culture medium

- Adding a layer of static liquid medium on top of a semi-solid medium (George, 2008)
- Explants take nutrients from both lower and upper layers
- Used successfully in *Pyrus* shoot multiplication enhanced by adding a liquid layer of culture medium on top of solid (Viseur, 1987)
- Use of water alone resulted in hyperhydricity, chlorosis, and decrease in shoot quality



Double phase medium, benzylaminopurine (BA) level, node number





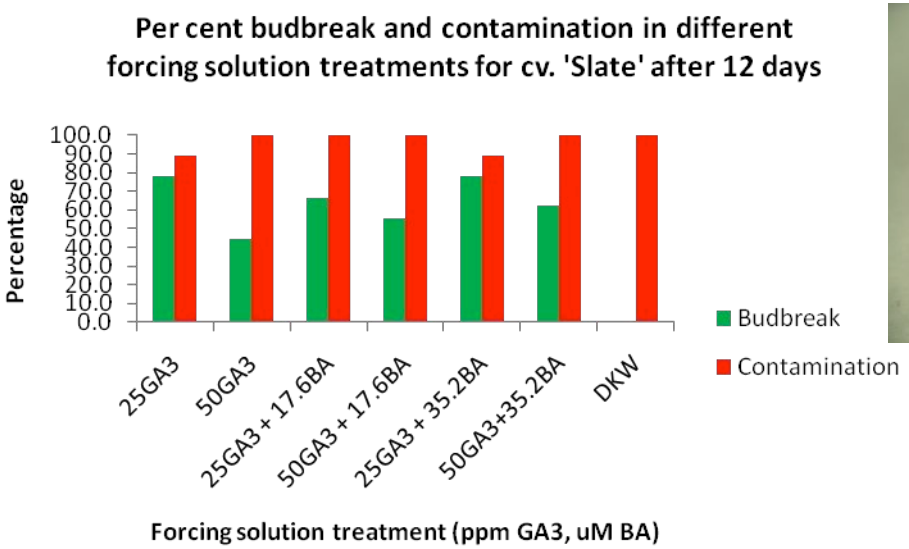
A **B** **C** **D**

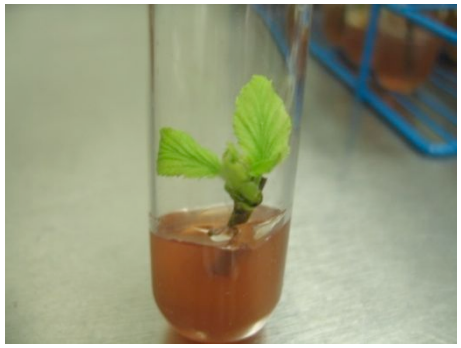
Hybrid cv. ‘16’ explants at 60 days grown in double phase media in boxes **A** and **B** and in solid media only in boxes **C** and **D**.

Effect of Forcing Solution

- Hybrid hazelnut shoots (hardwood cuttings) from previous season harvested in October 2010
- Cold dormancy for minimum 80 days at 4^{o}C
- See surface disinfection procedure
- Placed in individual test tubes with different treatments
- Forcing solution 2% sucrose + 200 mg/L 8-hydroxyquinoline citrate
- FS + two levels of GA_3 and/or BA
- Every three days –solution replaced, basal portion recut
- Ercisli (2001) used different forcing solution treatments on *C. avellana* x *C. americana* hybrids to determine time require to budbreak and amount of shoot elongation

Effect of Forcing Solution



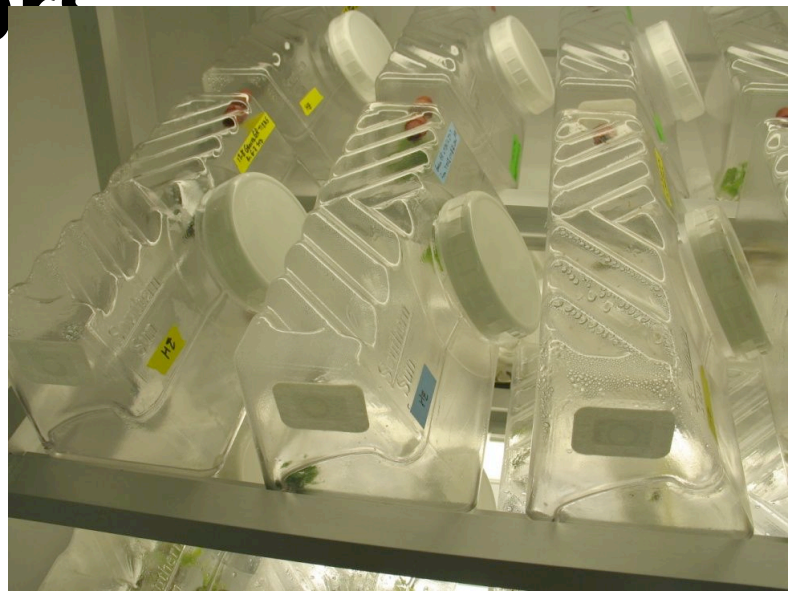


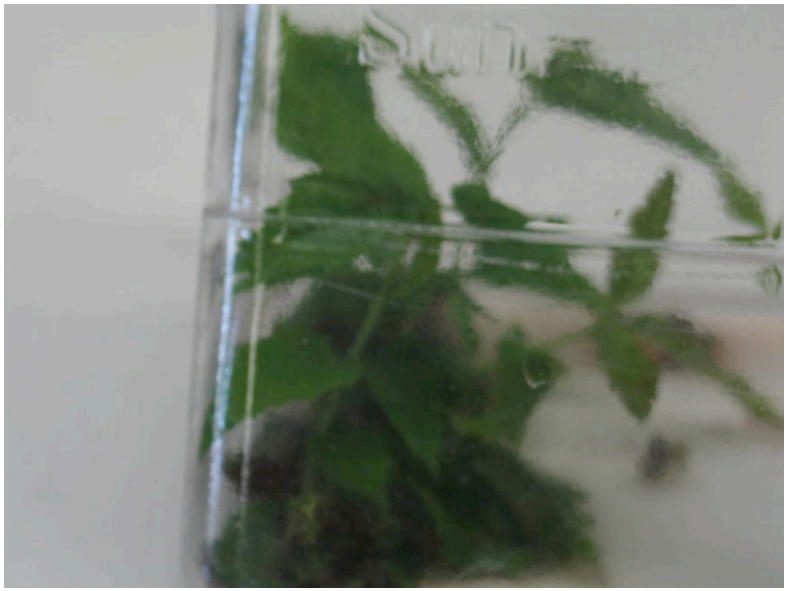
cv. 'Slate' from forced outgrowth in basal media

- 25ppm GA_3, 25ppm GA_3 + 35.2uM BA most effective
- Use of FS not effective for establishment of hazelnut explants due to 100% eventual contamination rate in most treatments, even with redisinfection procedure
- Require repeated disinfection to outrace fungi and bacteria
- Field cuttings from shoot canopy **not** a viable source for stock plant material

Potential for growth in bioreactors

- Liquid media using rocker system
- Hyperhydricity and quick proliferation of contamination potential issues

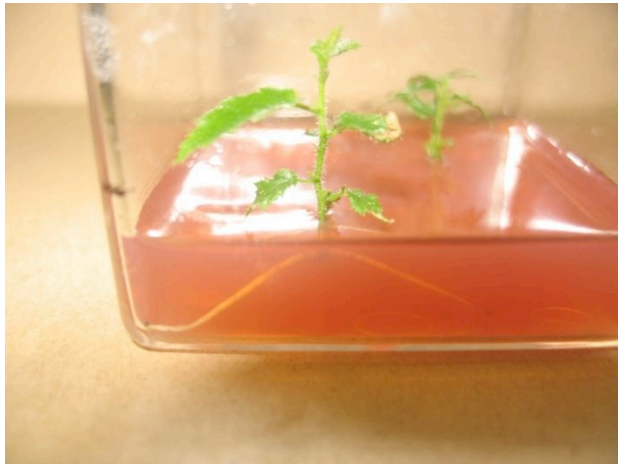




Stage III: Rooting - Effect of PGRs

- Initial results with IBA 0.5, 2.5, 5.0 mM and control
- 5.0 uM IBA lead to greatest number of plants exhibiting roots and number of roots but no root hairs
- 2.5 and 0.5 uM IBA longer roots with fine root hairs
- Use of rooting powder/solution or rooting *in vivo* i.e. Jiffy propagating plugs may be an alternative to rooting *in vitro*

Roots in 5.0 uM IBA cv. 'Geneva'



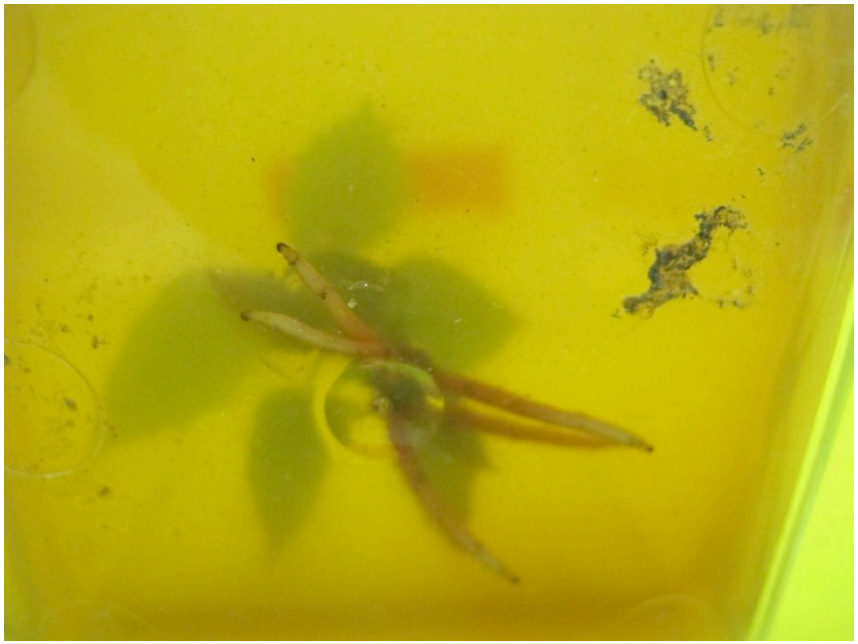
Roots in 2.5 uM IBA cv. 'Geneva'

Effect of PGRs on rooting

cv. ‘Geneva’ explants grown in multiplication medium, no BA, GA_3, three IBA treatments + control

Roots initials within 10 days in both 2.5 uM and 5.0 uM IBA

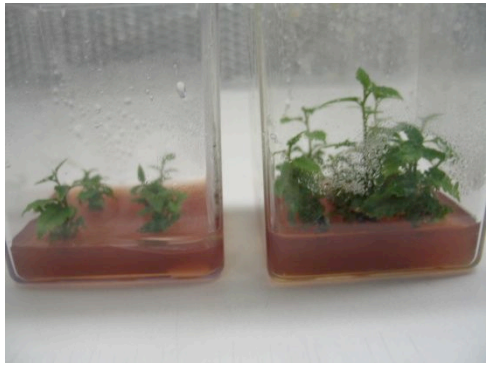
cv. ‘Geneva’ 2.5 uM IBA 21 days



cv. ‘Geneva’ 5.0 uM IBA 21 days

Research results

- Initiation and multiplication protocols established
- **Best stock plant source - greenhouse-grown**
- **EDDHA -effective iron source**
- **Nodal development and node number related to foliar quality *in vitro***
- Two-phase media can increase proliferation
- **Rooting achieved *in vitro* with IBA**



Future directions for research

- Continued optimization of culture media for specific cultivars
- Optimization of growth conditions
- Rooting *in vitro* with auxin, *in vitro* with no auxin, or *in vivo*
  - Effect on root morphology, number and length of roots and root hairs
  - Implications for *ex vitro* survival rates
- Standardization of acclimatization protocol

Thank you

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