CLONAL PROPAGATION OF WALNUT ROOTSTOCK GENOTYPES FOR GENETIC IMPROVEMENT 2008

Wesley P. Hackett, Chuck Leslie, Bruce D. Lampinen, Nicolas Manterola, Soussan Hirbod, and Gale McGranahan

ABSTRACT

We produced approximately 3500 liner-sized plantlets of 26 genotypes for greenhouse screens and for growing in nurseries to a size large enough for grafting and use in orchard trials. In addition, over 350 liner plantlets of 13 lines transformed for resistance to crown gall were produced for greenhouse re- tests for susceptibility to gall formation. A field plot was established at UC Davis to test the horticultural characteristics of the transgenic putatively crown gall resistant lines and controls. Root pruning of newly rooted microshoots was introduced on a trial basis in our micropropagation production process to improve root system structure of resulting liner plantlets. Survival of plantlets was reduced only slightly in *ex vitro* rooted plantlets but substantially in *in vitro* rooted ones. An experiment showed that leafless liner-sized plantlets of most clones can retain high viability after nine months storage including two months at 42-45ºF and seven months at 33ºF. Rooting hardwood cuttings of seedling trees less than two years old in pots showed promise as a relatively rapid (six months) method for clonally propagating plants for re-testing for crown gall resistance. A root grafting experiment showed that providing heat to the base of root grafts may not be necessary for successful rooting of most rootstock clones. The largest orchard trial to date, consisting of five clonal rootstocks and a seedling rootstock, all grafted to Chandler, and own-rooted Chandler, was planted in San Joaquin County. Trees were grown at Suchan Nursery for establishment of an orchard trial at Cilker Orchards in Yolo County.

GOAL AND OBJECTIVES

The goal of this project is to provide the California walnut industry with new clonal rootstocks selected or designed to combat the most threatening pests and diseases. The overall objective is to devise clonal methods of propagation for candidate genotypes and to provide clonal plantlets so that they can be evaluated in greenhouse and field replicated disease and pest challenge tests.

PROCEDURES AND RESULTS

Propagation

We have continued to use three approaches to clonally propagate candidate rootstock genotypes with nematode, crown gall, *Phytophthora*, or blackline tolerance or resistance:

A. Tissue culture micropropagation with *in vitro* and *ex vitro* rooting of microshoots.
B. Dormant hardwood cuttings on bottom heated beds.
C. Bench grafted root cuttings during the dormant season.

Tissue culture micropropagation: We produced approximately 3500 liner plantlets of 26 genotypes for replicated disease and pest screening tests by Greg Browne, Dan Kluepfel and Mike McKenry and for growth and grafting in nurseries for subsequent orchard trials (Table 1). We also produced about 350 liner plantlets of 13 lines transformed for putative resistance to crown gall plus non-transformed control lines for use in greenhouse screening re-tests for crown gall resistance by Dan Kluepfel (Table 1). This past year's production, plus hold-over plantlets from the previous year's production, gives us an inventory of about 5000 liner plantlets ready for immediate use in replicated disease and pest resistance re-tests and in nursery row growth and grafting for orchard trials (Table 2). Based on an experiment we reported in 2007 which showed improved root structure of liner plantlets that had been severely root pruned at the time of planting in liners, we introduced root pruning into our production process on a trial basis. Table 3 shows that root pruning at the time of planting reduced survival of both *ex vitro* and *in vitro* rooted plantlets. The reduction in survival was quite small (68 to 63%) for *ex vitro* rooted plantlets but larger (71 to 51 %) for *in vitro* rooted plantlets. We don't have an explanation for why survival was reduced more by root pruning of *in vitro*, as compared to *ex vitro*, rooted plantlets. Based on these results we will continue root pruning as a standard practice but also try to figure out why root pruning may reduce survival more for *in vitro* than *ex vitro* rooted plantlets.

Cold storage of liner size plantlets derived from micropropagation is advantageous for at least three reasons: 1) storage conserves greenhouse bench space 2) storage at temperatures of 42-45ºF provides the chilling necessary for maximum growth potential when plantlets are planted in the nursery row or orchard site 3) storage allows year-around laboratory production while permitting choice of the most desirable time for nursery row or orchard planting.

The length of time plants can be stored has been restricted because plantlets stored at temperatures around 42-45ºF begin to grow after about five months. Because storage occurs with only very low light, the shoots that form are etiolated and the growth depletes the stored nutrients required for nursery or orchard establishment and growth. In an effort to extend the length of storage time possible without shoot growth, we performed an experiment using a lower storage temperature (33ºF). After an initial eight week storage period at 42-45ºF with short days and low light to cause leaf abscission and to put the plants in a dormant condition, the plantlets were transferred to a cold room with no light at 33ºF. Plants were then transferred to greenhouse conditions after five, seven and 10 months storage at 33ºF to assess survival and growth. Table 4 shows that the survival of nine different clones was very high overall after five and seven months of storage (89 and 87%, respectively) at 33ºF but dropped off markedly to 35% after 10 months storage. Two clones (Burbank and UX2) appeared to be losing some viability after storage for seven months as their survival in the greenhouse had dropped off to 60 and 40 % respectively, whereas the other clones still had survivals of 80 to 100%. These results indicate that leafless liner-size plantlets of most clones can retain high viability after nine months of storage, including two months at 42-45ºF and seven months at 33ºF. The reason that plantlets lost viability after nine months is not known but it could be due to desiccation of the leafless shoots, since the humidity at 33ºF is quite low. We are currently doing an experiment to see if we can reduce loss of viability at 33ºF by storing the plantlets inside plastic garbage bags to reduce water loss and desiccation.

Liner plantlets from cold storage were re-potted into avocado pots (1½ gallon) and then grown in the greenhouse to provide actively growing 3/8-1/2 inch diameter trees for use in screens for crown gall resistance by Dan Kluepfel. Forty five plants of seven transgenic lines that had shown crown gall resistance in previous screens and control plants were provided. Thirty plants each of AZ025, RX1, Vlach and VX211 were also provided for an inoculation dosage test along with 120 AX1, 80 AX2 and 35 AX3 plants for experiments on methods of wounding and inoculation for improved crown gall screens. These plants were grown and screening tests were performed in a Plant Science greenhouse specifically managed for crown gall screening. Liner plants were also grown on to a size large enough for *Phytophthora* screening. A total of 670 plantlets of 17 genotypes from cold storage were re-potted in Cetap 1½ liter pots and grown further in the greenhouse to provide actively growing 1/4-3/8 inch diameter trees for use in screens for *Phytophthora citricola* and *P. cinnamomi* resistance. Screens were performed by Greg Browne in a USDA greenhouse.

Fully chilled liner plantlets of 10 clones (16 to 36 plants each) were used in a nursery experiment to test the effect of root pruning on the quality of root systems produced in the nursery row. Plantlets from 1½" diameter x 7" deep Tree Tubes were planted in a nursery row in May with root systems intact or the lower 2/3 of the root balls pruned away. Resulting trees will be grafted with Chandler in 2009 and dug in 2010 at which time the quality of root systems will be evaluated.

Hardwood cuttings: Hardwood cutting material of RX032, *Juglans cathayensis* #21 and UZ229 from the original mother trees at Kearney Research and Extension Center was received from Mike McKenry in mid-January 2008. Because these trees had not been pruned to stimulate vigorous shoot growth, the material did not yield many cuttings of the quality that have the best chance of rooting. In addition we collected cutting material from the *J. cathayensis* #21 mother-tree at the Wolfskill Experimental Orchard. Hardwood cuttings of all four genotypes were made, treated basally with 8000 mg/l potassium indole butyric acid and placed on a bottom-heating bed at 27C. About 25% of the cuttings of *J. cathayensis* #21 and UZ229 rooted but none of the RX032 or *J. cathayensis* mother tree rooted. We were able to provide Mike McKenry with five rooted-cutting trees of *J cathayensis* #21 and 10 rooted-cutting trees of UZ229 for a nematode resistance trial at Kearney. Subsequently the mother trees at Kearney were pruned so that more high quality cutting material will be available in 2009.

Hardwood cuttings were also used to clonally propagate for re-testing 95 open pollinated seedlings from mother trees representing nine species from the National Clonal Repository that had no galls or small gall formation 15 months (two growing seasons) after inoculation with a virulent strain of *Agrobacterium tumefaciens.* A total of about 300 cuttings were made in early February and treated as described above. A total of 89 rooted cuttings were grown on in avocado pots for re-testing by Dan Kluepfel's laboratory. Rooting of cuttings from seedling trees in pots less than two years old shows promise for a relatively rapid (six months) method for clonally propagating plants for re-testing for crown gall resistance. Use of hardwood cuttings reduces the number of genotypes that need to be propagated by *in vitro* micropropagation which is initially slow to produce plantlets and labor and resource intensive.

Bench grafting root cuttings: A small root-grafting experiment was performed to compare rooting of root pieces when the temperature was kept at 78ºF at either the graft union only or at both the graft union and the base of the root piece. As shown in Table 5, rooting was as good or better for three of four clones tested when heat was provided at the graft union only. With AZ025, a sparse rooting clone, providing heat to the base did improve rooting somewhat. It may be that the heating cable at the graft union provides a temperature at the base, even though probably lower than that at the graft union, high enough to promote rooting. This suggests that the optimum temperature for rooting may be lower than that for graft union healing (however, no temperature measurements have been made to confirm this). These results do indicate that providing heat to the base of root grafts may not be necessary for successful rooting of most clones and may even be detrimental.

Due to concern that the root pieces used to make root grafts have been in contact with field soil and that the wounding necessary to the process could increase the incidence of crown gall on the resulting nursery trees, we evaluated 129 root grafted trees after two years in the nursery row. Seven of the 129 trees had galls (5.4%) at the site of the graft union. This is a very high incidence of crown gall and may indicate that root grafting can exacerbate nursery problems with crown gall. However, of the six rootstock clones used for the root grafts, WIP3 trees had five of the seven galls detected. The frequency of galls on WIP3 trees was 36%. The frequency on the other five clones was 1.8% which is still quite high. The high incidence for WIP3 trees is based on a small sample but other data based on inoculation of WIP3 trees with a virulent strain of *A. tumefaciens* also indicate that it is quite susceptible to crown gall formation.

Field Trials: Currently established clonal rootstock field trials are summarized in Table 6.

Fully chilled liner plantlets (100 each) of Vlach, VX211, RX1 and Burbank were provided to Suchan Nursery for growing to sufficient size for planting as dormant bare-root trees in an orchard trial to be established at Cilker Orchards in Yolo County in 2009 and grafted with Howard scions.

The largest orchard trial of clonal rootstocks to date was planted this year in San Joaquin County under the direction of Joe Grant. Chandler-grafted nursery trees using five rootstock clones and seedling paradox were dug in January 2008 and planted along with own-rooted Chandlers.

A one acre field plot at UC Davis was planted with transgenic, putatively crown gall resistant lines and controls for assessment of horticultural characteristics.

Nursery Propagation and Commercialization: We are prepared to provide cultures of microshoots to any laboratory or nursery that wants them for licensed production of plants. We can also provide microshoots of Vlach, a public domain clone, to any laboratory or nursery that wants to produce it. The appendix includes a list of laboratories presently licensed for *in vitro* production of clonal rootstock selections and sale of clonal rootstock plantlets as liners for nursery or orchard planting.

Table 1. Greenhouse survival of rooted clonal microshoots

	# Alive	# Total	% Survival
Paradox			
AX1	544	700	78
AX2	18	22	82
AX3	21	29	72
Burbank	40	67	60
DAR	174	309	56
GZ2	6	11	55
GZ3	164	227	72
JX1	24	54	44
MW1	42	113	37
Px1	54	76	71
RX1	713	1105	65
UX1	27	78	35
UX2	23	36	64
UZ 229	198	367	54
UZ1	50	75	67
UZ2	21	46	46
Vlach	145	282	51
VX211	525	733	72
XZ1	10	19	53
	2799	**4349**	**64.4%**
CLRV Tolerant			
WIP2	95	284	33
WIP3	407	806	50
WIP4	81	197	41
WIP6	76	230	33
	659	**1517**	**43.4%**
English			
Chandler	14	102	14
Chandler	14	102	14
Hartley	38	96	40
Howard	7	35	20
RL	5	8	63
Tulare	4	12	33
	82	**355**	**23.1%**

Table1 (cont.). Greenhouse survival of rooted clonal microshoots

	# Alive	# Total	% Survival
Crown gall resistant			
J1 12A	2	11	18
J1 13A	11	18	61
J1 15B	0	1	0
J1 19A	7	9	78
J1 1A	1	7	14
J1 20A	3	10	30
J1 2A	1	4	25
J1 3A	2	3	67
J1a control	212	320	66
J21a control	5	9	56
J21b control	36	71	51
J21b control	36	71	51
RR4 12A	3	3	100
RR4 control	24	26	92
	343	**563**	**60.9%**
Modified phenolics			
CR1	1	17	6
CR1 control	8	21	38
CR1 PPO 12	0	23	0
CR1 PPO 12	0	23	0
CR1 PPO 2-1	0	12	0
CR1 PPO 40-1-1	5	30	17
CR1 PPO 42-6	0	27	0
CR1 PPO 42-6	0	27	0
CR1 PPO 42-6	0	27	0
CR1 PPO 42-6	0	27	0
CR1 PPO 96-1-2	3	52	6
	17	**286**	**5.9%**
Black			
W17	**1**	**6**	**16.7%**
Wingnut/hybrids			
WNBxGRZ 1a	31	97	32
WNxW 10.05 b	3	4	75
	34	**101**	**33.7%**
Total	**3935**	**7177**	**54.8%**

Table 2. Inventory of fully acclimated clonal rootstock plants currently available for use in disease and pest resistance trials and field trials in 2009

	Genotype	Number of plants inside EH 45 F Cold Room - Older than 5 months (Updated: 12/15/08) *	Number of plants inside EH 45 F Cold Room - younger than 5 months (Updated: 12/15/08)	Plants recently introduced to EH 45F Coldroom Date 12/15/8 (Survival Data 12/08/08)	2nd Group Mann Lab Plants (Date 5/16/08)	3rd Group Mann Lab Plants (Date 6/11/08)	4th Group Mann Lab Plants (Date 7/16/08)	5th Group Mann Lab Plants (Date 8/26/08)	6th Group Mann Lab Plants (Date 9/8/08)	7th Group Mann Lab Plants (Date 10/29/08)	8th Group Mann Lab Plants (Date 10/29/08)	9th Group Mann Lab Plants (Date 12/11/08)	Plants regrowing in SIB	FIRST CHILL in EH 45 F COLD ROOM (Date 11/14/8)	TOTAL PLANTS (DATE 12/15/08)
1	**84-121**	0	0											1	**1**
1	**AX1**	0	85	18	50	58	31	39	57	30	108	89	40	4	**609**
1	**AX2**	2	30		11								23	1	**67**
1	**AX3**	0	33		4	13							10	15	**75**
1	**AZ025**	0	0											15	**15**
1	**AZ2**	0	0											2	**2**
1	**AZ3**	0	0											3	**3**
1	**Burbank**	0	5		11								86	1	**103**
1	**DAR**	0	46	5		24	27	16	36	6	7	13	2	10	**192**
1	**GZ2**	6	0											21	**27**
1	**GZ3**	0	92		35	34	28				10		10	21	**230**
1	**JX1**	0	29										137		**166**
1	**MW1**	0	9			14	4	1	5	14	1			14	**62**
1	**PX1**	0	75										9	4	**88**
1	**RX1**	61	176	25		81	12	5	37	57	11	124			**589**
1	**UX1**	0	34		9		7		3				12		**65**
1	**UX2**	0	12										5	149	**166**
1	**UZ 229**	0	17	14	4	76		21	23	4	13	1	26	1	**200**
1	**UZ1**	0	61		31	17							100	4	**213**
1	**UZ2**	0	29		8								14		**51**
1	**Vlach**	18	45	15			8	2	14	15	3	12	5	4	**141**
1	**VX211**	8	36	5	27	98	22	34	78	32	110	45			**495**
1	**XZ1**	0	17										101	10	**128**
2	**WIP1**	0	0											1	**1**
2	**WIP2**	0	36	18			12	11	15		3	1	1	10	**107**
2	**WIP3**	0	231	21		43	31	15	14		11	31	10		**407**
2	**WIP4**	0	43				13	7		15		1			**79**
2	**WIP6**	0	24	9			6	2	2	14		14		2	**73**
3	**CR1 control**	0	1				4	1					7	1	**14**
3	**CR1 PPO 40-1-1**	0	0									5			**5**
3	**CR1 PPO 96-1-2**	0	0							2		1			**3**
6	**CR**	7	0							4		4			**15**
6	**Hartley**	0	10					16				11			**37**
6	**Howard**	0	0				1	1				5		1	**8**
6	**Tulare**	0	0									4		1	**5**

Table 2 (cont.). Inventory of fully acclimated clonal rootstock plants currently available for use in disease and pest resistance trials and field trials in 2009

7	**J1 12A**	**0**	**2**												**2**
7	**J1 13A**	**0**	**6**	**5**											**11**
7	**J1 16A**	**0**	**0**										2		**2**
7	**J1 17A**	**0**	**0**										2		**2**
7	**J1 19A**	**0**	**10**												**10**
7	**J1 1A**	**0**	**1**												**1**
7	**J1 20A**	**0**	**3**												**3**
7	**J1 2A**	**0**	**1**												**1**
7	**J1 3A**	**0**	**2**												**2**
7	**J1 6A**	**0**	**1**												**1**
7	**J1a control**	**0**	**6**				55	30	109		1				**201**
7	**J21a control**	**0**	**0**										35		**35**
7	**J21b control**	**0**	**0**		11	19							125		**155**
7	**RR4 10A**	**0**	**0**										2		**2**
7	**RR4 12A**	**0**	**0**	**3**											**3**
7	**RR4 2A**	**0**	**5**											2	**7**
7	**RR4 2C**	**0**	**0**										1		**1**
7	**RR4 3A**	**0**	**9**												**9**
7	**RR4 4A**	**0**	**0**											1	**1**
7	**RR4 6C**	**0**	**0**										3		**3**
7	**RR4 7A**	**0**	**0**										13		**13**
7	**RR4 8A**	**0**	**1**												**1**
7	**RR4 9A**	**0**	**0**											6	**6**
7	**RR4 control**	**0**	**56**		22								16	6	**100**
9	**Rawlins**	**0**	**0**					5							**5**
9	**W17**	**0**	**0**											6	**6**
10	**WNBxGRZ 1a**	**0**	**0**	**4**					9	6					**19**
10	**WNxW 10.05 b**	**0**	**1**					2						3	**6**
	TOTAL	**102**	**1280**	**142**	**223**	**477**	**261**	**208**	**402**	**199**	**278**	**361**	**797**	**320**	**5050**

Table 3. Survival of plantlets produced from in vitro or ex vitro rooted microshoots with roots pruned or unpruned at time of planting

	Ex vitro rooted						In vitro rooted						Combined		
	Unpruned			Pruned			Unpruned			Pruned					
	Alive	Total	% Survival	Alive	Total	% Survival	Alive	Total	% Survival	Alive	Total	% Survival	Alive	Total	% Survival
Paradox															
AX1	131	139	94	138	198	70	10	11	91	265	352	75	544	700	78
AX2	18	22	82										18	22	82
AX3	21	27	78				0	2	0				21	29	72
Burbank	40	67	60										40	67	60
DAR	15	34	44	105	134	78				54	138	39	174	309	56
GZ2				6	11	55							6	11	55
GZ3	99	135	73	35	46	76	4	4	100	26	42	62	164	227	72
JX1	24	43	56							0	11	0	24	54	44
MW1	16	43	37	24	60	40	1	1	100	1	9	11	42	113	37
Px1	50	61	82							4	15	27	54	76	71
RX1	193	287	67	340	480	71	7	11	64	152	295	52	713	1105	65
UX1	21	55	38	3	15	20				3	8	38	27	78	35
UX2	18	27	67				5	9	56				23	36	64
UZ 229	53	98	54	66	148	45	19	22	86	46	78	59	198	367	54
UZ1	46	67	69				4	8	50				50	75	67
UZ2	21	46	46										21	46	46
Vlach	23	37	62	67	145	46				47	91	52	145	282	51
VX211	79	105	75	193	261	74	6	7	86	247	360	69	525	733	72
XZ1	10	19	53										10	19	53
	878	**1312**	**67%**	**977**	**1498**	**65%**	**56**	**75**	**75%**	**845**	**1399**	**60%**	**2799**	**4349**	**64%**
CLRV Tolerant															
WIP2	2	7	29	60	106	57	0	1	0	24	132	18	95	284	33
WIP3	82	102	80	81	157	52				228	488	47	407	806	50
WIP4	4	11	36	45	88	51	1	4	25	31	94	33	81	197	41
WIP6	11	11	100	21	31	68				44	186	24	76	230	33
	99	**131**	**76%**	**207**	**382**	**54%**	**1**	**5**	**20%**	**327**	**900**	**36%**	**659**	**1517**	**43%**
Total	**977**	**1443**	**68%**	**1184**	**1880**	**63%**	**57**	**80**	**71%**	**1172**	**2299**	**51%**	**3458**	**5866**	**59%**

Table 4. Greenhouse survival of 3 month old walnut rootstock plantlets after storage for 8 weeks at 42ºF followed by storage at 33ºF for various time periods.

	% Survival after cold storage		
Genotype	5 Months @ 33ºF	7 Months @ 33ºF	10 Months @ 33ºF
AX1	84	100	0
AX2	91	100	54
Burbank	100	60	0
JX1	100	100	50
UX2	83	40	29
UZ1	71	80	32
UZ2	100	100	0
Vlach	100	100	57
XZ1	84	100	14
Total	89	87	35

Table 5. Influence of heating the bases of grafted root pieces on the rooting success of four rootstock clones grafted to Chandler.

	Both Graft Union and Base Heated		Only the Graft Union Heated	
Genotype	% Rooting	Root No.	% Rooting	Root No.
AZ025	94	++	94	+
RX1	100	+++	100	+++
Vlach	100	++++	100	+++++
VX211	100	++++	100	+++++

Whip and tongue grafts made, bases of root pieces treated with 8000 mg/L K-IBA, and stuck in moist wood shavings on 2/25/08. Thermostatically controlled heating cables in the wood shavings provided a temperature of 78ºF at the graft union. Data taken 4/9/08.

Table 6. Current Clonal Rootstock Field Trials.

County	Grower	Genotypes	Date Established	Comments
Butte	Deseret	RX1, AZ2	2006	New orchard Grafted with Chandler
Sutter/Yuba	Conant	RX1,VX211, Serr, Vlach	2007	New orchard
Sutter/Yuba	Double Nut	VX211, AZ2, NZ1, JX2	2006	Phytophthora Replants
Solano	Lester	GZ1, AZ2, JX2	2005	Replants - water issues
Contra Costa	Tennant	WIP2, WIP3	2006	Blackline tolerant New orchard
Calaveras	Gotelli	RX1,VX211, AX1, AX2, WIP3, GZ1, AZ2, Px1, UX2, 84-121, AX3, GZ2, NZ1, GZ3, JX2, wingnut	2004	Phytophthora plot New orchard
San Joaquin	Dondero	RX1, VX211, AZ2, NZ1, JX2	2005	Phytophthora Replants
San Joaquin	Taylor	RX1, VX211, AZ2, NZ1, JX2	2005	Phytophthora Replants
San Joaquin	Taylor	WIP3, WIP5, WIP6	2005	Blackline tolerant New orchard
San Joaquin		RX1, VX211, WIP3, AZ025, Vlach, own-rooted Chandler	2007	New orchard Grafted with Chandler
Merced	Crane Jr.	AZ2, AZ3, NZ1, JX2, AX1, GZ1, Px1, AZ1, UX1, GZ2, WIP3, UX022	2005	Phytophthora Replants
Kings/Tulare	Headrick	Vlach	2007	New orchard Grafted with Chandler

Laboratories Licensed to Produce Liners of UC Clonal Rootstock Selections

California Seed and Plant Lab

License: RX1, VX211

Test agreement: Px1

Duarte

License: RX1, VX211

Test agreement: WIP3, AZ025

North American Plants

License: RX1, VX211

Test agreement: WIP2, WIP3, WIP6

ProTree

License: VX211, RX1

V-Tree

License: VX211, RX1

Test Agreement: WIP3

VitroTech

License: RX1, VX211

Test Agreement: WIP2, WIP3