Title: Developing rooting protocols on non-*P. communis* pears and promising wild pear germplasm: a prerequisite study for in vitro screening Armillaria resistance

PI: Sugae Wada, Oregon State University, Horticulture Department, Corvallis, OR

ABSTRACT

In vitro culture and rooting of pear rootstock cultivars is important for producing planting materials for the pear industry. Difficulties in traditional propagation, as well as micropropagation of promising dwarf pear rootstocks, has long been delayed the introduction of improved cropping systems for pear orchards. Development of new pear micropropagation media now makes it possible to produce all types of scion pears in vitro, recent development of improved growth media for pear rootstock (PRS medium) provided needed information for improving rootstock culture as well as rooting. In micropropagation of fruit crops, adventitious root formation of *in vitro* grown shoots is a major obstacle, so efficient rooting is a critical step for economical production. Our prior study (2013 CPAB project) established an efficient rooting protocol for the *P. communis* species but then *P. betulifolia* species (OPR-113, 114 and 260) did not respond at all to the protocol. This year's project explored exclusively with non-*P. communis*. Three *P. betulifolia* cultivars with six promising wild species were investigated in this project (CPAB 2018). Moderate success of rooting with three *P. betulifolia* (62-83%) and high success (80-100%) for the six wild species were reported in this study.

OBJECTIVES

- Developing non-*P. communis* rootstocks *P. betulifolia* species (*P. betulifolia*, 'OPR-113', 'OPR-114', and 'OPR-260').
- Initiating and establishing healthy in vitro shoot cultures for the selected wild pear germplasm sub culturing on PRS medium for the rooting trial (Table 1).
- Testing rooting potential of diverse in vitro shoot cultures (non-*P. communis*: four *P. betulifolia* species and 6 diverse wild germplasm).
- Developing one or more improved rooting protocols for each species.
- Results of this study will be used for identifying *Armillaria* resistance by in vitro screenings and further support genomic analysis for the pear improvement studies.

PROCEDURE

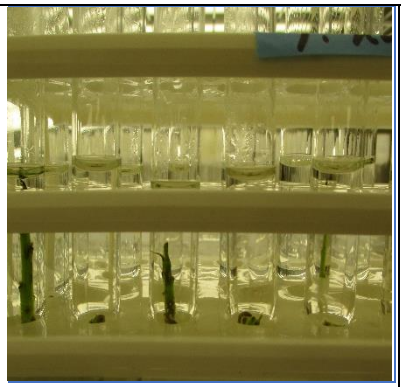
Plant materials.

P. betulifolia species: *P. betulifolia* 'OPR-113', 'OPR-114', and 'OPR-260' in cultures in OSU and others received from the NCGR in vitro germplasm collection those cultured on PRS medium were used. One received *P. betulifolia* (CPYR 649.001) in vitro germplasm was found out to be an identical genotype with *P. betulifolia* 'OPR-113' (CPYR 655.001), therefore

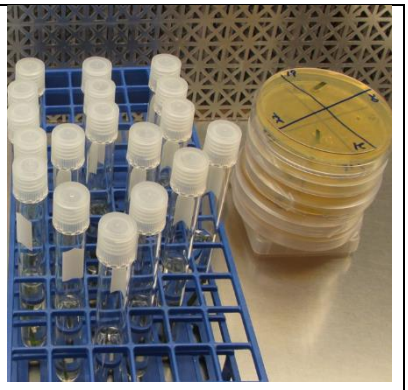
it is not used for this study.

Wild pear initiation: At the time of USDA-ARS germplasm order for the in vitro wild pears for this study, three genotypes, *P. pashia* 'Variolosa', *P. korshinskyi*, and *P. salicifolia,* were not available, therefore fresh cuttings from the NCGR field collection were received, and newly initiated from the cuttings. After the sterilization processes with bleach, washing, and shaking on G-10 Gyratory shakers (New Brunswick Scientific, N.J. USA) for the explants, shoots were soaked in liquid detection medium in small test tubes for 7 days. All contaminated cultures found at this stage were discarded, only a small number of tubes that contained clean cultures were moved onto PRS medium. Additional bacteriological plates were used for screening each month for 3 times then, again only clean shoots were multiplied for 5-6 months at OSU tissue culture lab (Figure 1).

Cuttings from the NCGR field pear collections



Pear shoots soaked in liquid contamination detection medium



VISS plate screening before planting onto PRS medium

Figure 1. *Wild pear initiation process into the sterile in vitro cultures*

NCGR germplasm received in Starpacks: Starpacks were submerged in 20% bleach solution for 10 minutes and dried under the laminar hood for 20 minutes. Ethanol (70%) sprayed on all over the surface before making an incision in the middle of the cell well with a sharp blade and take the pear shoots out from each cell well. Then cut the living plant parts into large pieces that contains internodes, then those pieces were planted on PRS medium in a GA7 container. Heathy out growth from the initial internodes were cut and move them onto fresh PRS medium after sub-culturing for 4 weeks and multiplied for 2-4 months until acquiring enough plant materials to test.

Culture conditions. Three genotypes, *P. pasha* 'Variolosa', *P. salicifolia* and *P. korshinskyi*, were not available at the time of germplasm orders from the National Clonal Germplasm Repository, fresh collected from the cuttings of NCGR field pears in April 2018. Others were received one or two cell wells of the Starpacks. Explants were grown in tissue culture containers (Magenta® GA-7, Magenta Corp., Chicago, IL, USA) with 40 ml medium per container. The base medium used was Pear Rootstock Medium (Wada and Reed 2017,

unpublished) composed of MS (Murashige and Skoog 1962) mineral salts modified to have 2.5× the MS level of mesos (Ca, Mg, P), and with per liter: 2.5 mg thiamine, 250 mg inositol, 30 g sucrose, 4.4 µM N^6-benzyladenine (BA), 0.7% agar (A111, PhytoTechnology Labs) adjusted to pH 5.7 and autoclaved for 20 min at 120 °C and 20 psi. Chemicals were sourced from PhytoTechnology Laboratories, (Shawnee Mission, KS, USA) and medium stock solutions were prepared in house. Shoots were transferred to new medium every 4 weeks. Cultures were grown at 25°C under a 16-h photoperiod with an average 80 µM $m^{-2}s^{-1}$ irradiance provided by a combination of cool- and warm-white fluorescent bulbs.

Table 1. Taxonomy, origins, and plant ID of wild pears

Taxonomy	CPYR	Plant Name	Origin
Pyrus pashia	411.001	Naspati	Nepal
Pyrus pashia	589.002	Variolosa	Uncertain
Pyrus korshinskyi	2522.004	Jalal-Abad 94011	Afghanistan
Pyrus regelii	2587.001	*P. regelii* Boraldy River Forest	Kazakhstan
Pyrus salicifolia	2382.001	*P. salicifolia* Russia	Russian Federation
Pyrus spinosa	634.001	*P. amygdaliformis* Turkey	Turkey

Rooting experiments. Four-week fully matured pear shoots sub-cultured on PRS were used for in vitro rooting experiments. Rooting % were evaluated after 4 weeks or 8 weeks depending on genotypes. Based on the preliminary study, selected auxin concentrations and PEG as a solvent Polyethylene glycol 400 (PEG 400, Sigma Aldrich, St. Louis, MO, USA diluted with deionized (DI) water to a 40% concentration.

1) Dipping method (Fig. 2): Auxins, naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA), 5 mM or 10 mM NAA+ 5 mM or 10 mM IBA combined, or 10, 20, 30, or 35 mM IBA) dissolved in the 40% PEG solution and filter sterilized (Nalgene 150 mL, Thermo Scientific, NY, USA). Shoots were dipped for 5 seconds in each rooting solution with nine shoots per container and four containers per treatment (n=36). Treated shoots were planted on PRS medium with no BA. For the first week treated containers were enclosed in brown paper bags or covered with aluminum foil creating a dark period and for the following 3 weeks grew under normal light condition (80 µM $m^{-2}s^{-1}$ irradiance.

2) Growth medium (PRS with no BA) contained a range of IBA in the lower range of concentrations (0.15, 0.2, 0.25, 0.3, or 0.5 mg/L) and later was raised to the higher range of concentrations (0.1, 0.15 or 0.2 g/L) for the several difficult-to-root genotypes, (other conditions as same as 1).

Data: Rooting rates were evaluated after 4 weeks for well responding genotypes *P. pashia* 'Variolosa', *P. korshinskyi*, *P. regelii*, and *P. spinosa*, or eight weeks for slow and the difficult-to-root genotypes *P. pashia* 'Naspati', *P. betulifolia* 'OPR-113' 'OPR-114', and 'OPR-260'.

Fig. 2. *Dipping method applied on P. spinosa at 20 mM IBA dissolved in 40% Polyethylene Glycol and planted on PRS medium with no BA resulted 100 % rooting after 4 weeks.*

RESULTS

With the three cultivars of *P. betulifolia,* moderate rooting success was induced in a narrow range of exceptionally high concentrations of IBA contained in PRS medium (no BA) in this study (Table 2). Also, 4 weeks after the treatments there were no genotypes that produced roots. *P. betulifolia* also required a longer period (~8 weeks) compared to *P. communis* cultivars (~4 weeks) as seen throughout prior rooting experiments. Two *P. betulifolia* species "OPR-113 and 114' exhibited symptoms of hypersensitivity to NAA, (somewhat less harmful effects on 'OPR-260'). Any time when NAA was added into the auxin mixtures in the dipping solution, after a week from planting, plants showed damage in leaf chlorosis, browning or eventually were dead at 4 weeks in severe cases (data not shown). Therefore, the number of IBA concentrations contained in PRS medium were mainly explored throughout study and *P. betulifolia* species rooted moderately well, although with weak root hairs (Table 3).

Wild pears newly collected in April 2018 all showed excellent growth and multiplication on PRS medium (Fig. 3). The rooting study required a high volume of explant numbers, but about three months of subculture provided enough materials. As well as, all three genotypes resulted in high successes of rooting rates in the series of IBA concentrations contained in PRS medium after 4 weeks (Table 3B).

P. regelii was the only species that produced abundant soft callus (Fig. 4) that completely covered delicate root hairs, thus this made difficult to see/confirm the root tissues. When rarely it produces visibly large/thick root tissue (tap root like) then plants dropped leaves, started to show chlorotic, weakened, and eventually died in 1-2 months. Later, even with additional care to supply the liquid PRS medium (BA a half strength) onto the agar surface they still died. One genotype, two cell wells of Starpack received *P. pashia* 'Naspati' in vitro germplasm, a rosette form growth habit, was slow growing, difficult to multiply to the needed amount of the explants, and sometimes exhibiting latent bacterial contamination from one cell well, so it could not be included in several treatments. Only a few showed short root hairs the first time in the highest IBA concentration (0.2 g/L IBA) at the final stage of experiments (Table 3B). However, this concentration (0.2 g/L IBA) appeared as excessive with such visual symptoms, thus testing with 0.15 g/L has been setup and currently on going. The data will

be taken January 20, 2019. Fig. 5 presents a combined total result of the rooting for all tested genotypes in this study.

Table 2. Dipping methods with IBA in solution (% rooted at 4 or 8 weeks)

Plant name	Cultivar	CPYR	10 mM*	20 mM	30 mM	35 mM	Sparklines
			(4 week)	(4 week)	(4 week)	(8 week)	
P. betulifolia	OPR-113	655.001	0	0	0	77	
	OPR-114	656.001	0	0	0	83	
	OPR-260	1379.001	22	96	-**	-	
P. pashia	Naspati	411.001	0	-	0	0	
P. spinosa		634.001	33	100	95	95	

***** *10 mM IBA concentration=0.203 g/100 mL in dipping solution*

****** *Not tested because of either explant availabilities (mainly 'Naspati'), or already highly rooted on the lower levels of IBA concentration*

Table 3A. Three *P. betulifolia* cultivars on PRS medium (no BA) with IBA (% rooted at 8 weeks)

P. betulifolia	5 mg	10 mg	15 mg	20 mg	25 mg	30 mg	0.1 g	0.15 g	0.2 g	**Sparklines**
OPR-113	0	0	33	33	33	39	55	*C**	62	
OPR-114	0	0	17	22	22	33	50	*C*	55	
OPR-260	10	10	35	35	50	50	83	*C*	83	

***** *Current, 8-week data will be taken after 4 more weeks (Jan 20, 2019)*.

Table 3B. Six wild pears on PRS medium (no BA) with a series of IBA concentrations (% rooted at 4 weeks)

Wild pears	5 mg	10 mg	15 mg	20 mg	25 mg	30 mg	0.1 g	0.15 g	0.2 g	**Sparklines**
P. pashia 'Naspati'	0	-*	0	-	0	-	0	*C***	11	
P. pashia 'Variolosa'	28	33	62	72	77	83	100	94	88	
P. korshinskyi	28	28	55	72	83	83	94	-	-	
P. regelii	22	22	44	44	62	77	83	55	*D****	
P. salicifolia	39	44	72	72	77	83	94	100	88	
P. spinosa	22	22	44	50	88	88	100	-	94	

***** *Not tested because of either explant availability, or already highly rooted on the lower levels of IBA concentration*

****** *Current, 8-week data will be taken after 4 more weeks (Jan 20, 2019)*

******* *Dead, shoots all had roots but dead at 8 weeks.*

Fig. 3. Three newly collected *P. korshinskyi*, *P. salicifolia*, and *P. pashia* 'Variolosa', in April 2018 from NCGR field plants all showed excellent initiation, growth/ multiplication on PRS medium (photos, left column), and highly rooted in several treatments. Rooting morphology showed in the photos at right column.

Fig. 4. Subculture of *P. regelii* out of the Starpack (photo, left) and it produced abundant soft calluses when treated for rooting (photo, right).

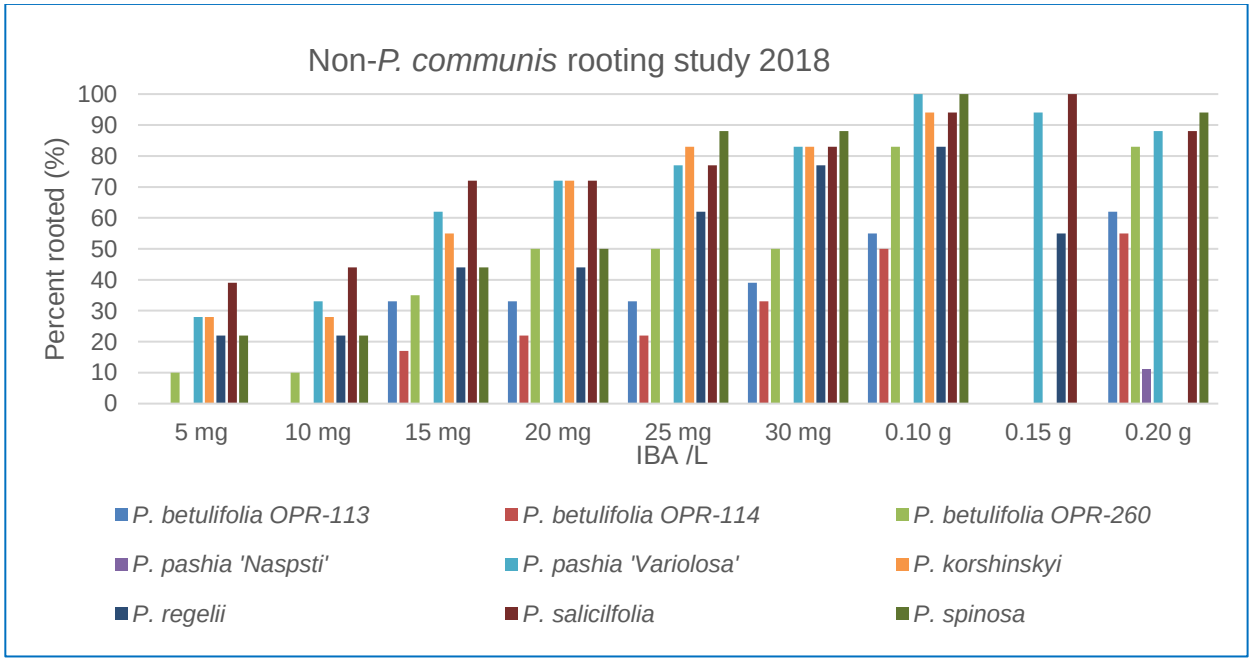


Fig. 5. *Combined rooting % graph for all of three P. betulifolia and six wild pears*

DISCUSSION

For the *P. betulifolia* in vitro rooting, those required unexpectedly high concentrations of rooting hormones compared to other woody crops such as hazelnut (normally 1 mg/L IBA). It not only exhibited a specific preference for IBA over NAA, but also showed a high negative sensitivity to the NAA. In this study, rooting of the three *P. betulifolia* cultivars was

moderately induced over a prolonged period of 8 weeks, however, the root tissues at this stage were still delicate and fragile. In order to have larger/thick root tissues for the evaluation of following *Armillaria* resistant screening, then those weakly rooted *P. betulifolia* species at 8 weeks should be additionally sub-cultured moving onto the fresh PRS medium with a half strength BA for 1-2 more months. Overall all but one of the new wild pears including newly collected from field plants (except *P. pashia* 'Naspati') produced satisfactory rooting rates in quality. *P. communis* 'Winter Nelis' (data not shown) was also added into this study because it did not root well in our prior *P. communis* rooting project. 'Winter Nelis' had 100 % rooting induced when dipped with 10 mM IBA at this time. *P. regelii* rooted well in 0.1g/L IBA, but did not well survive after rooting, thus it was difficult to maintain alive as rooted plants. However, this study will be partially supportive to start the new CDFA project dealing with 80 in vitro pears. There remain many pear species untested, thus still remaining unknown for their rooting abilities such as, *P. bretschneideri, P. calleryana*, *P. koehnei, P. pyrifolia* and *P. syriaca,* including all the hybrid species.