Walnut rooting results in September 2018 at the facilities of Industrial Plants Ltd.

Our research team at IndPl is currently doing large-scale experiments for the elaboration of an improved protocol for walnut in vitro rooting. This is necessary because the rooting protocol provided by Dr. Licea-Moreno from BN does not work at IndPl, although it works at BN.

Here at IndPl we encountered one major problem: the optimal amount of vermiculite per culture vessel (580 ml standard canning jar) was totally unknown. The amount of 8 g/vessel recommended by Dr. Licea-Moreno was not suitable. The amount of 100 ml root expression media added per each jar totally flooded the vermiculite layer at the bottom of the jar. Thus, the rooting percentages and the quality of the roots were very low, absolutely not suitable. Therefore, we tested several amounts of vermiculite/vessel for the root expression setup. At the beginning the quantities of 12-15 g/vessel were sometimes suitable, the media were absorbed into the vermiculite. On other occasions the vermiculite layer remained partially flooded after autoclavation. In the root expression stage the rooting percentages/vessel were totally random, between zero and 50 %, in most cases with just a few rooted plantlets/vessel. Due to these facts, our conclusion is that our vermiculite is very different from the one used at BN and there could be some random errors caused by the vermiculite absorbing water vapour from the air.

During the years of 2017 and 2018 we carried out extensive research for walnut in vitro rooting, as we mentioned earlier. As a result, now we have a two-stage protocol similar to the original one, the one used at BN. Also, we do not use a special elongation stage because the shoots that result from our current multiplication media (which also leaves much to be desired) are elongated and robust, suitable for rooting.

For walnut multiplication in order to obtain shoots suitable for the rooting stages we use the medium presented in Table 1, codenamed WM3 at IndPl, which we presented earlier.

Table 1. The composition of WM3 medium used at IndPl

|Components	Concentration
DKW BN powder	Full concentration, according to the recipe
$Ca(NO_3)_2$ x7H_2O	Full concentration, according to the recipe
Phloroglucinol	50 mg/l
BA	0.7 mg/l
Sugar	30 g/l
Gelrite	1.2 g/l

According to our experience, the optimal culture period on this medium is one month (30 days), after which the plant material must be subcultured either on the same medium for multiplication or cut and transferred to the root induction media.

The optimal period for root induction is strictly three days, not five. Even if there is just one day of delay there are deformations in the plant material in the root induction media.

The compositions of the root induction media and the new root expression experimental treatments are presented in Table 2.

The root induction stage was slightly improved, we use pre-sterilized plastic boxes in order to economize space, materials and manpower. Thus, the process can be performed faster, on-demand (Fig. 1).

The amount of root induction media/vessel was 330 ml, in one-liter plastic boxes. The optimal number of explants (walnut shoots) per vessel was 30.

The optimal number of shoots transferred to the root expression media is 15 shoots/jar. Thus, theoretically at least, if no material is lost, two jars of root expression media should be calculated for one box of root induction stage material.

After transfer to the root expression media, rooting took place in approximately three weeks after inoculation. The compositions of the root induction media and of the root expression treatments done at IndPl are presented in Table 2.

The amount of vermiculite added to the culture vessels (580 ml canning jars) used for the root expression stage was 18 grams, dispensed into the jars before adding the media. The pH was adjusted prior to adding Gelrite powder and then Gelrite was suspended using a stirrer; the suspensions were dispensed with syringes into the jars. Autoclavation parameters: 30 minutes at 121 ºC.

Table 2. The composition of root induction and root expression media tested at IndPl

		Treatments for root expression:	
Components	Walnut root induction medium concentrations	Treatment 1 concentrations	Treatment 2 concentrations
DKW macronutrients	Half-strength	Half-strength	Half-strength
DKW micronutrients	Full-strength	Full-strength	Full-strength
DKW iron	Full-strength	Full-strength	Full-strength
Vitamin B1	1 mg/l	1 mg/l	1 mg/l
Vitamin B6	0.5 mg/l	0.5 mg/l	0.5 mg/l
Nicotinic acid	0.5 mg/l	0.5 mg/l	0.5 mg/l
Glycine	2 mg/l	2 mg/l	2 mg/l
Sugar	40 g/l	-	40 g/l
Fructose		40 g/l	-
IBA	10 mg/l	-	-
Gelrite	-	1 g/l	1 g/l
Plant Agar	4 g/l	-	-

PH adjusted to 5.7

After one month (30 days) in the root expression media, the rooted as well as non-rooted plantlets were transferred ex vitro in greenhouse conditions, into various substrates presented below:

1) 100% peat;

2) peat:vermiculite 1:1 v/v ratio;

3) peat:vermiculite 3:1 v/v ratio.

The results obtained in our first two preliminary tests are presented in Table 3. In this setup the in vitro rooting was quite good as compared to our earlier results. However, the plants seemed to be underfed, there were some dead shoot tips and yellow leaves. Our hypothesis is that this happened because the root expression media were exaggeratedly diluted by the very high amount of vermiculite, which was more than double the amount in the original protocol. We present some comments on our observations in Table 4.

Table 3. The results of the first two root expression tests at IndPl in September 2018.

Experimental series/date	Treatment	Number of vessels/ treatment	Number of rooted plants	Total number of explants	Rooting percentage
1 / **27.8.2018**	1	15	149	197	75.634
	2	16	104	236	44.067
3.9.2018	1	9	84	112	75
	2	11	64	140	45.714

Meanwhile we performed another two tests for rooting, where the compositions of the root expression media were totally different, for example in one of them we used treatments of full-strength MS, WPM, QL with different concentrations of sugars and we are anxiously waiting for the results.

Table 4. Comments based on the observations of the experimental treatments of the first and second rooting series

Experimental series/date	Treatment	Comments
1 / **27.8.2018**	1/fructose	The roots on Treatment 1 (fructose) were not much different from the sugar treatment
	2/sugar	
3.9.2018	1/fructose	The roots were weaker than the sugar treatment
	2/sugar	Very strong roots

We are going to present pictures of the walnuts from our experimental treatments in Fig. 2.

Fig. 1. Walnut cultures in boxes on root induction media: A, B – cultures in boxes; C – shoots transferred for inoculation into root expression media.

Next we are going to present some pictures from the walnut in vitro rooting experiments and ex vitro transfer procedures for acclimatization. We present our ex vitro transfer procedures in Fig. 3. and some images of the ex vitro transferred walnut plants in Fig. 4. We are anxiusly waiting for the results of ex vitro acclimatization. So far, according to our observations in the last two weeks, there is no necrosis but there is no growth. All the rooted and non-rooted plantlets are alive and seem to be well and we hope for the best.

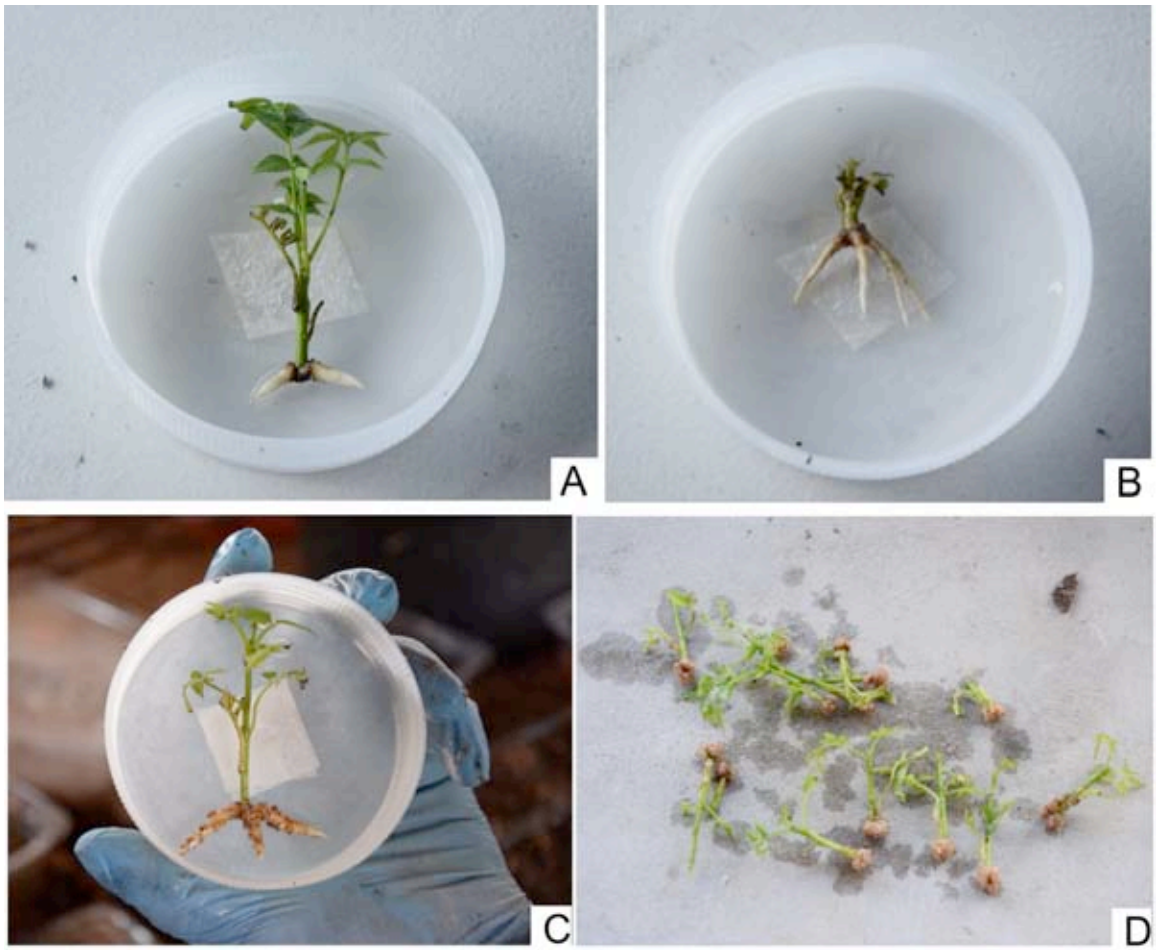

Fig. 2. A, B: plantlets from Treatment 1 (media with fructose), C: plantlets from Treatment 2 – media with sucrose, D: non-rooted plantlets

Fig. 3. A, B: machine for mixing the substrates for acclimatization and another machine for dispensing the substrates into cell trays at IndPl, C: preparing a small batch of substrate for the ex vitro acclimatization test, D: ex vitro transfer of walnut plantlets into cell trays with substrate

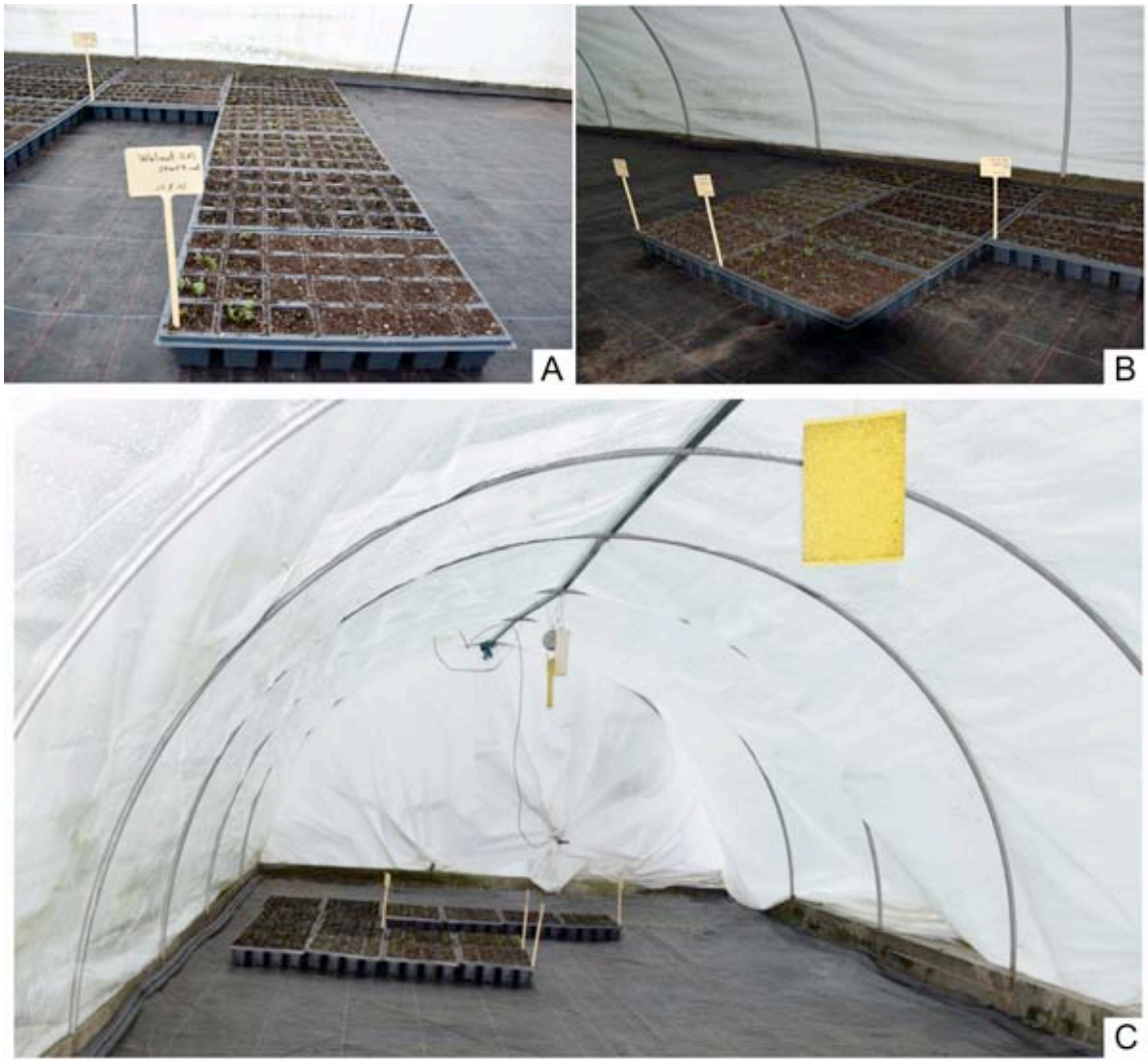

Fig. 4, A, B, C: Walnut plantlets transferred ex vitro for acclimatization in a greenhouse at IndPl