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REGENERATION OF *CUPHEA WRIGHTII* (PEYR 651) AND FERTILE *C.WRIGHTII* X *C. TOLUCANA* HYBRIDS FROM LEAF EXPLANTS

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Abstract: Callus was obtained from leaf explants of *Cupea wrightii* and *Cuphea wrightii* x *Cuphea tolucana* hybrid plants, and the plants were later regenerated. *C. tolucana* explants were capable of forming callus, but not of regenerating. In order to obtain callus from *C. wrightii* and the hybrid plants, the addition of BAP to the medium was necessary, whereas in the case of *C. tolucana* auxin was needed. The regeneration of the plants did not require auxin, and both forms (*C. wrightii* and the hybrids) regenerated in the same medium. The regeneration yield came to around 12 plants from the callus of one harvest. Some of the callus from the hybrids was subjected to colchicine treatment, which increased the number of fully fertile regenerants from 1% to almost 20%.

Key Words: *Cuphea*, Interspecies Hybrids, *In Vitro* Cultures, Regeneration, Callus.

INTRODUCTION

The seeds of some species of *Cuphea* contain 18 - 42% fat, and of their fatty acids, c. 80% are medium-length chain saturated fatty acids (MFA) (C12:0, C10:0, C8:0). Only coconut palms and oil palms contain fat with a similar MFA make-up. Many *Cuphea* species also have low soil and water requirements. This has created interest in these species as potential sources of fats of medium-length chain saturated fatty acid composition. However, these species require several genetic alterations to be accepted as agriculturally useful plants. The

results of numerous studies seem to indicate that *in vitro* cultures and interspecies hybrids could be a promising source of variability. To make them truly useful, it is necessary to find high-yield methods of regeneration, and in the case of the hybrids - additional methods, which will increase their fertility, as the hybrids of the species presented here are almost completely infertile. To this end, attempts to induce high-yield regeneration for these forms and their fertile hybrids have been undertaken. Thus far three *Cuphea* species have been replicated via *in vitro* culture - *C. wrightii* [1, 2], *C. glutinosa* [3] and *C. ericoides* [4].

MATERIALS AND METHODS

Plant material

In the experiments, the following species were used: *Cuphea wrightii* Peyr 651 and *Cuphea tolucana* A. Gray 629, and their F1 hybrids, obtained at the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań. Both of these species are entirely self-pollinating (cleistogamic). For both hybridization and in the *in vitro* work, lines bred for three generations were used.

In order to obtain non-sterile explant donors, seeds were put into Petri dishes with wet blotting paper inlays. Imbibition was done at room temperature. After 12 hours, the seed coat was removed, and the seeds were transferred onto fresh blotting paper. Further germination took place at a temperature of around 26°C. After 2 - 3 days, when the cotyledons were open, the seedlings were potted in pots with a peat substrate. The pots were placed in a phytotron at a temperature of around 24°C and an exposure of 2000 Lx. The exposure time was 16 hours per day.

Material for further research was taken after around 30 days of growth. The leaves from the third and fourth nodes from the top of the main stem (four or five leaves per plant), and the internode from above the third and fourth nodes served as the source of explants. All the *C. tolucana* x *C. wrightii* hybrids from which explants were to be taken died, so explants were only taken from *C. wrightii* x *C. tolucana* hybrids. The latter formed few seeds, but they were considerably differentiated genetically.

In order to obtain sterile explant donors, the hairs on the seed surfaces were destroyed, as per Olejniczak and Przybecki [5]. The seeds were then sterilized in 6% sodium hypochlorite for 20 minutes, and then rinsed three times in distilled, sterilized water. After the removal of the seed coat, seeds in sets of three were placed in 1.5 l beakers in a medium containing $^{1}/_{2}$ of the macro- and microelement composition as per Murashige and Skoog (MS) [6], without vitamins and hormones, with 0.7% agar as the gelling agent. The explants were taken as described above.

In vitro culture

In order to optimize regeneration conditions, three experiments were performed. The first used eleven different media (Tab. 1), and two explant types (leaf and stem) taken from plants grown in sterile conditions. The second used the above-mentioned media and explants taken only from the leaves of plants grown in non-sterile conditions. In these two experiments, the *C. wrightii*, *C. tolucana* and hybrid lines were used. The third was an additional attempt to obtain *C. tolucana* regeneration, and in this experiment additional media (K_{12} - K_{17}) were used.

The samples taken were sterilized in 6% sodium hypochlorite for 15 minutes, and then rinsed three times in distilled, sterilized water. The edges, tip, and base of each leaf and the main nerve were thrown out, and 3 x 4 mm leaf slivers (10 - 15 per leaf, i.e. around 50 from each plant) from the central parts of each leaf, and 5 mm long sections of the stem were placed in Petri dishes of 8 cm diameter on an agar medium containing as a substrate MS + 200 mg/l edamine + 7 g/l agar.

Tab.1. Growth regulator composition in media for *Cuphea wrightii, Cuphea tolucana* and their hybrids (concentration in mg/l)

Growth regu-lators	Medium																
	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	K_{11}	K_{12}	K_{13}	K_{14}	K_{15}	K_{16}	K_{17} 1/2MS
2.4D	1.0	0.1	1.0	1.0	0.1	0.1	0.1	0.01	0.01	0.01		0.1	0.1	0.1			0.02
6-BAP			0.1	1.0	0.1	1.0	10.1	0.01	1.00	10.0	1.0					0.8	0.20
IpAd												0.8					
Zeatin													1.0				
Kinetin														1.0	0.2		
2.4.5.T																1.2	
IAA															1.0		

The medium had a pH of 5.6 [7]. The hormone combinations and concentrations are given in Table 1. The explants were placed on the medium right side up. For each combination (combinations of medium type, explant type, genotype), 50 explants were laid out - 5 per dish. For the first 4 weeks, this material was kept in the dark at a temperature of 26°C. Passaging was performed more or less at four-week intervals. From the first passage on, the callus was exposed to light of 800 Lx intensity. The exposure period was 16 hours per day. Some of the callus from the non-fertile hybrids was subjected to colchicine treatment after around two months. The callus was soaked for 30 minutes in colchicine solution made up according to Jensen [8] (10ppm GA_3, 2% DMSO, 0.1% colchicine, 2 -

3 drops/100ml Tween 20), and then rinsed four times in distilled, sterilized water. The callus was then laid out on fresh medium.
After around four weeks, observation of the growth of the callus was performed. Measurements of the intensity of the regeneration were also done, expressed as the number of regenerated stems/callus/harvest.
Regenerated stems of 1.5 – 5 cm were cut in sterile conditions and moved to jars of 0.5 l with an agar gel medium (0.7%) containing $^{1}/_{2}$ MS without hormones. Around 8 plants were placed in each jar. Root formation took place in the same exposure and temperature conditions as the regeneration. The jars with rooted plants were moved (after 1 - 2 weeks) to a greenhouse and left there for one or two days for acclimatization purposes. Then seeds were collected from the plants.

RESULTS

The conditions for regeneration

Regeneration occurred after the callus phase, which was characterized by certain specific traits. Due to this, *in vitro* culture will be described in two steps, dealing with the callus phase separately from the regeneration itself.
The results of the experiments performed to establish the conditions for callus formation and plant regeneration (shoots and roots) are given in Tables 2 - 4. The number of explants presented there differs considerably from the number laid out. This was a result of the large number of endogenous infections (from the explants), even in the case of those plants which where grown in sterile conditions. It was not possible to use ”harsher” sterilization conditions because of the considerable damage to the leaf blade caused by the action of hypochlorite. Other sterilization methods which were tested in the initial experiments were less effective.

Callus formation

There are at least a few characteristics typical of *Cuphea* callus formation (Tabs. 2 - 4). One obvious trait is the lack of callus formation (with the exception of 7% of the *C. tolucana* explants in experiment II) for leaf explants in media without cytokinins (media K_1 and K_2). A similar sensitivity to the absence of auxin was displayed by *C. tolucana* (medium K_{11}). The shoot explants (in experiment I) did not form callus on media K_1, K_7 and K_8. Shoot explants from *C. wrightii* and the hybrids did not form callus on media K_3 and K_{10}. It was noticeable that, with the exception of a few cases (medium K_3), a higher percentage of callus-forming explants came from plants grown in sterile conditions than from those in non-sterile ones. In some cases, these differences were very large, e.g. in the case of *C. tolucana* and the hybrids on medium K_{10}, or *C. tolucana* on medium K_9.

Tab. 2. Results of experiments on callus formation and plant regeneration from leaf and shoots explants obtained from plants C*uphea wrightii, Cuphea tolucana* and their F_1 hybrids, grown in sterile conditions. Abbreviations: C. w. (*C. wrightii*); C. t. (*C. tolucana*); H (F1 C.w. x C.t. hybrids); L (leaves); P (shoots); K (roots); < (less than); + (positive reaction); callusing expl. % = callusing expl. No./No. of explants x 100; callus size scale (arbitrary) 0 - 3

Medium No.	Species	Number of explants		Callusing expl. No.		Callusing expl. %		Size of callus		Plant parts regeneration		
		L	P	L	P	L	P	L	P	P	K	P+K
	C.w.	25	15	0	0	0	0	0	0			
K_1	C.t.	21	18	0	0	0	0	0	0			
	H.	17	21	0	0	0	0	0	0			
	C.w.	28	14	0	3	0	21	0	1			
K_2	C.t.	22	16	0	7	0	44	0	1			
	H.	20	13	0	5	0	39	0	<1			
	C.w.	28	31	14	0	50	0	2	0			
K_3	C.t.	24	18	18	3	75	17	2	1			
	H.	21	14	13	0	62	0	3	0			
	C.w.	18	25	14	11	75	44	3	1			
K_4	C.t.	27	48	11	11	40	61	3	1			
	H.	25	19	10	9	40	47	3	1			
	C.w.											
K_5	C.t.	26	20	26	0	100	0	2	0			
	H.	19	19	18	6	95	32	3	1			
	C.w.	30	18	30	18	100	100	1	1			
K_6	C.t.	22	18	15	6	67	33	1	2			
	H.	27	15	24	5	88	33	2	1			
	C.w.	31	21	31	0	100	0	<1	0			
K_7	C.t.	21	25	21	0	100	0	<1	0			
	H.	24	20	22	0	92	0	<1	0			
	C.w.	20	28	20	0	100	0	3	0			+
K_8	C.t.	18	20	18	0	100	0	3	0			+
	H.	19	22	19	0	100	0	2	0			+
	C.w.	28	18	16	18	56	100	2	2			
K_9	C.t.	29	24	29	24	100	100	2	2			
	H.	21	17	15	17	71	100	2	3			
	C.w.	26	21	9	0	35	0	<1	0			
K_{10}	C.t.	24	18	24	6	100	33	<1	<1			
	H.	27	20	27	0	100	0	<1	0			
	C.w.	32	25	15	19	48	76	2	2	+		
K_{11}	C.t.	21	25	0	14	0	67	0	1			
	H.	29	22	17	18	60	77	3	2	+	+	+

Tab. 3. The results of experiments on callus formation and plant regeneration from leaf explants obtained from plants of *Cuphea wrightii*, *C. tolucana* and their F1 hybrids, which were grown in non-sterile conditions. Callus size scale 0 - 3 and abbreviations as for Tab. 2.

Medium No.	Species	Number of explants	Callusing expl. No.	Callusing expl %	Size of callus	Plant parts regeneration		
						P	K	P+ K
	C.w.	18	0	0	0			
K_1	C.t.	15	1	7	1			
	H.	15	0	0	0			
	C.w.	15	0	0	0			
K_2	C.t.	21	0	0	0			
	H.	18	0	0	0			
	C.w.	18	16	88	1			
K_3	C.t.	30	30	100	2			
	H.	22	22	100	3			
	C.w.	36	10	30	3			
K_4	C.t.	26	17	67	1			
	H.	22	7	32	3			
	C.w.	36	18	50	3			
K_5	C.t.	24	8	33	2			
	H.	30	14	47	2			
	C.w.	24	20	84	3			
K_5	C.t.	30	30	100	2			
	H.	25	17	68	3			
	C.w.	30	18	60	2			
K_7	C.t.	30	14	46	3			
	H.	24	14	58	3			
	C.w.	30	12	40	3			
K_8	C.t.	18	10	56	2			
	H.							
	C.w.	36	20	56	2			
K_9	C.t.	24	8	33	1			
	H.	28	15	54	2			
	C.w.	30	12	40	2			
K_{10}	C.t.	30	8	26	<1			
	H.	21	9	43	2			
	C.w.	20	5	25	1	+		
K_{11}	C.t.	20	0	0	0			
	H.	22	6	27	2	+	+	+

The distribution of the frequency of explant callus formation in the populations varied. In the sterile-grown populations, callus formed with high frequency on explants growing on media K_5 - K_{10} (with a few exceptions), while in the case of non-sterile-grown populations, media K_3 and K_6 gave such high callus formation

frequency - these media had hormones in reversed proportions. Relatively few explants formed callus on medium K_{11}. It is worth noting that on this medium there were twice as many callus formations on plants grown in sterile conditions as on those from non-sterile ones.

The *Cuphea* callus also has characteristic traits. Its growth, as shown in Tables 2 and 3, was not particularly fast, and was often even very slow. This growth was significantly faster for callus from leaves than for that from stems. The callus from stems was soft and turned brown quickly. The leaf callus was generally hard and aged more slowly than the stem callus. The aging symptoms included the appearance of white ”fluffy” callus, the appearance of anthocyanin-dyed cells (this trait does appear very early, without other symptoms of aging), or the browning or blackening of the callus. Blackening of the callus often occurred without a browning phase, and was accompanied by large amounts of an oily-looking secretion. The callus generally became green after being exposed to light. There were some small differences between the callus coming from different species. The *C. tolucana* callus could be described as more often than not soft or of medium hardness. It significantly more rapidly became ”fluffy”, turned green to only a slight degree, and aged early. The callus from the hybrids was the hardest, and was very green. It often grew in an interesting way, giving flat, disc-like plates. Its visible, often concentric rings suggested that the growth could have occurred evenly in thin layers around the whole circumference of the callus.

Regeneration

Regeneration was only obtained on medium K_{11}, i.e. on a medium which did not give the fastest callus growth, but which gave a very hard, green callus which blackened relatively late in its oldest parts. During passaging, these blackened parts were cut out. Regeneration only occurred in the case of *C. wrightii* and the hybrids. Research into what type of regeneration had occurred was not carried out, but everything indicates that it was organogenesis. Regeneration from the callus from the sterile-grown plants only occurred for one explant of *C. wrightii*, with a yield of 2.3 plants/harvest/callus, while in the case of the hybrids, 3 explants regenerated with an intensity of 3.1 plants/harvest. For the non-sterile-grown explant donors, four calluses of each of the two plant forms regenerated, which comes to 70 - 75% of the total number of callus-forming explants. More or less four explants per donor plant formed callus, and the regeneration yield came to 13.3 plants/callus/harvest for *C. wrightii* and 12.5 for the hybrids. Regeneration was not obtained for *C. tolucana* despite the use of a variety of hormone combinations (due to the presented lack of any reaction, combinations lacking in one of the hormones were not used) (Tab. 4).

There were no significant differences between the amount of regeneration for *C. wrightii* and for the hybrids, but there were qualitative differences. In the case of *C. wrightii*, rhizogenesis and stem regeneration never occurred together on

the same medium, nor on the same callus, whereas co-occurrence of these two processes was normal for the hybrids. It is important to note that rhizogenesis itself did not appear to have any influence on the regeneration of the stems.
Seeds were collected from the plants obtained. Among the hybrids coming from calluses which had not been subjected to colchicine treatment, less than 1% were fertile. This was probably an effect of spontaneous polyploidisation during the culturing. Treating the callus with colchicine increased the fully fertile plant yield to 20%.

Tab. 4 Callus formation for *Cuphea tolucana* leaf explants on media with different growth regulators. Callus size scale 0 - 3

Medium	No. of explants	Callusing expl. No.	Callusing expl. %	Callus size
K_{12}	31	17	58	3
K_{13}	37	25	68	2
K_{14}	36	27	75	1
K_{15}	30	21	70	2
K_{16}	32	22	69	2
K_{17}	31	0	0	0

DISCUSSION

The main aim of this research was: first to establish the conditions for regeneration, and then to establish if – and to what extent – *in vitro* culture explants of somatic tissue from two *Cuphea* species and their hybrids could be a good source of genetic variability. For this reason, an explant type was chosen which, as could be expected from the literature [1, 7], would fulfill the following two basic requirements: it would give the highest possible number of regenerated plants and a relatively high variability [9, 10], and it would be straightforward from a methodic-technical point of view. The time and position of explant collection were determined by the speed of the plants' development. Both species, *Cuphea wrightii* and *Cuphea tolucana*, grow relatively slowly in the initial phase of their development, and develop small leaves. Under the conditions set, after about a month of growth, the plants were developed enough to be considered both productive enough and of sufficiently high quality to serve as a source of explants.
To obtain callus and regeneration, first the media as per Janick and Whipkey [1], with a broader set of hormones, were used. However, regeneration was not obtained. Only after casein hydrolate was replaced with endamine, and i-inosytol with m-inosytol, in the amounts used in media for cucumbers [7], was

regeneration obtained, but only for *C. wrightii* and the hybrids. *C. tolucana* did not regenerate despite the use of additional sets of hormones.

The results of research into the formation of callus for *Cuphea wrightii*, *Cuphea tolucana* and their F1 hybrids lead to the conclusion that there are differences between species within the *Cuphea* genus as regards the reaction of the leaf explants to the media used. Such a phenomenon was already described for other plants [11, 12, 13, 14, 15]. The leaf explants from the above-mentioned species and hybrids of *Cuphea*, regardless of the conditions they were grown under (sterile, non-sterile), required the presence of cytokinin in the medium to initiate the callus formation process. *C. tolucana* required auxin in addition to this. Characteristics of the hybrids in culture, such as their requirement for hormones to initiate callus formation, the consistency of their callus and the speed of its growth, and their hormone requirements for regeneration, were similar to or the same as those of the mother form (*C. wrightii*). It is difficult to say with certainty what kind of dependence this is (nucleic, cytoplasmic), as cultures of reverse hybrids were not successfully made. Not all the traits of the hybrids in the culture were the same as those of the mother form. An example of a trait where difference occurred was in the percentage of explants forming callus. This value was very variable, and it is difficult to define what type of expression this is.

The conditions under which the explant donors grow seem to have a very significant influence on callus formation. The explants from the plants grown in sterile conditions formed callus significantly more frequently than those from plants grown in non-sterile conditions. *Cuphea* is characterized by a generally slow growth rate of callus (an arbitrary assessment, not in measured units, but based on a comparison with other species such as cucumber and tobacco), and does not seem to be related to the percentage of the explants that form callus. Evidence of that was the growth of callus on media K_{10}, K_8 and K_4, from leaf explants from a plant grown in sterile conditions. On the first of the three, the frequency of callus formation was high, while the growth of the callus was not significant. On the second, both the formation frequency and the growth level were high, while on the last, the formation frequency was rather low, and callus growth level was high.

The ability to regenerate was only observed on the medium without auxin. The value of this trait was the same for *C. wrightii* and the hybrids. The callus formation frequency on this medium was not the highest observed (25 - 60%, depending on the growth conditions of the donor plants and the genotype; *C. tolucana* did not form callus at all on this medium). The intensity of callus growth was not high either. However, this callus was very hard, turned green easily, and aged quite slowly. Stem regeneration began quite early, within the second week of the explants being laid out, and kept up through 15 subsequent passages, without losing intensity over time. The regeneration yield came to an average of ten to twenty plants per callus per harvest, which gave a few hundred

to over a thousand regenerants from one explant over a 60-week period. Such productive regeneration only occurred for explants from plants grown in non-sterile conditions, whereas for sterile-grown plants, 2 - 3 plants per harvest were obtained. This indicates that there is no relationship between the explants' ability to form callus and the stem regeneration yield from those explants. With such a high regeneration yield, it would be possible to obtain more than five thousand regenerants from one plant in one year. This productivity could be at least doubled, were it possible to eliminate infection. Compared to species like *Nicotinia tabacum* [14], *Glycine max* [16], *Medicago truncatula* [17], or *Cucumis sativus* [7], this is a high regeneration yield. Any addition of auxin inhibited regeneration. These results do not fully concur with the results obtained for *C. wrightii* regeneration by Janick and Whipkey [1], who found the highest regeneration yield on media with BAP and 2.4-D.

The described relationship of regeneration yield and medium suggested a correlation between yield and the ratio of cytokinin to auxin in the medium. On the other hand, Sultanbawa *et al.* [3] obtained regeneration from a callus from a leaf of *Cuphea glutinosa* on an MS medium with a hormone composition similar to that used here. *C. glutinosa* is an annual with a fatty acid composition similar to that of *C. wrightii*. Janick and Whipkey [1] also managed to obtain regeneration from stem-derived callus, which was not done successfully here. One of the sources of this difference could have been differences in the growth conditions of the explant donor plants. This factor could have had a significant influence on the regeneration processes (as shown by the results of this research), but exact details about the sampling position on the plant and the age of the plants were not given by those authors - the plants are simply described as having been mature. This makes it impossible to precisely establish the condition of the explant donors. The studied form could also have been a different sub-species or variety. Research on the influence of factors such as the growth conditions of the explant donors, the sampling location, or the age of the plants was already conducted before"?][1, 14, 16, 18, 19, 20]. Janick and Whipkey [1] took their explants from the stems of regenerated plants, which could have contributed to more productive regeneration. Nolan *et al.* [17] showed that in the case of *Medicago truncatula*, regeneration yield was significantly higher when the leaf explants were taken from R_0 or even R_1 plants, suggesting that an increased regeneration potential may be transferred through the seeds.

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