In Vitro Regeneration From Different Types Of *Cannabis* Explants

Samuele Paganelli[1,2], Flavia Fulvio[1], Francesca Pia Macchia[2], Nadia Guerini[2], Laura Righetti[1], Roberta Paris[1*]

[1] CREA, Centro di Ricerca Cerealicoltura e Colture Industriali, via di Corticella 133, 40128 Bologna

[2] Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, Via G. Campi 103, 41125 Modena

*corresponding author: roberta.paris@crea.gov.it

INTRODUCTION

To reduce environmental impact while maintaining food security and ensuring sustainability in agricultural production, it is necessary to adopt resilient agricultural practices and promote biodiversity, exploring available plant resources for industrial purposes. Hemp (*Cannabis sativa* L.) can address several agricultural challenges and provide food products such as seeds and derivatives, as well as fibers and active biomolecules with various industrial applications. However, legal restrictions related to the presence of the psychoactive phytocannabinoid Δ9-THC in hemp flowers have significantly limited not only cultivation but also research and development of *in vitro* cultivation techniques, including regeneration, which is a prerequisite for the application of TEA. Although legal difficulties are still present, interest in this crop is increasing in Europe and worldwide, as is research in the field of *in vitro* culture. In the context of the CaRiFIT2022 project (Hemp and Italian Supply Chain Research 2022, https://carifit.crea.gov.it), CREA - CI in Bologna is committed to optimizing *in vitro* regeneration protocols for the application of CRISPR-Cas9 technologies for functional genomics studies and genetic improvement.

MATERIALS and METHODS

Regeneration from juvenile tissues

Plant materials: Commercial seeds (Fig. 1A) of Eletta Campana, Fibrante and Felsinea sterilized with an optimized protocol (3% H_2O_2 for 24h + final wash with 2.5% NaClO) and germinated *in vitro* according to Galán-Ávila et al. (2020).

Methods: Seedlings of about 10 days (Fig.1C) were dissected into three portions: hypocotyl, cotyledons, and true leaves. Each tissue was cultured in Petri dishes containing previously autoclaved regeneration media with different hormonal combinations, described in Tab. 1, partly chosen from literature. The basis of the regeneration medium composition, according to Galán-Ávila et al. (2020), consisted of 1/2 MS basal salts and vitamins (Murashige & Skoog, 1962) + 1.5% (w/v) sucrose + 8 g/L agar or alternatively 3.5 g/L Gelrite®. The pH was adjusted to 5.70 with KOH before sterilization at 121°C in an autoclave.

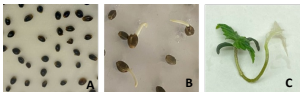


Figure 1. *Seeds, sprouted seeds and hemp sprouts of cv. Felsinea*

Table 1. *Composition of regeneration media tested.*

Code	Plant growth regulators (mg/L)	References
0	No plant growth regulators	Galán-Ávila et al., 2020
1	TDZ (0.4) + NAA (0.2)	Chaohua et al., 2016
2	Zeatin riboside (2.0)	García-Fortea et al., 2020
3	Meta-Topolin	this work

Explants were kept in a BINDER KBW 400 growth chamber, illuminated with cold light fluorescent lamps L 18W/865, at 22°C ± 1°C and a photoperiod of 16 hours of light. Explants were monitored periodically for 5 weeks of culture. Data were analyzed by R studio as in Galán-Ávila et al. (2020).

Regeneration from mature tissues

Plant materials: Sterile leaves and internodes from micropropagated shootlets of the genotypes V13, V20, V2 and V24 (Fig.2), maintained on DKW/Juglans medium supplemented with MS vitamins in a BINDER growth chamber have been used as starting material.

Figure 2. *Baby food jars with micropropagated cannabis shootlets.*

For all the regeneration tests, MS basal media supplemented with MS vitamin mixture was used, using agar 8g/L as gelling agent. Experiments were conducted in the BINDER KBW 400 growth chamber with the conditions described before.

Test 1. Leaf explants (560) and internodal stem sections (632) of V13 genotype were tested on eighteen media supplemented with TDZ (0.5-0.3-0.1 mg/L) and M_T (1.2-0.8 or 0.4 mg/L) combined with NAA (0.5-0.25-0.1 mg/L). 296 leaf explants and 280 stem sections of V20 genotype were tested only on the combinations of M_T and NAA. Callus color, consistence and dimension was evaluated as in Kumar et al. (2015) and regeneration events (roots, shootlets) recorded.

Test 2. Internodal stem sections of three genotypes (V2, V20 and V24) were tested on four media supplemented with 2,4D and diversified for the cytokinin (BAP, MT, TDZ, ZTR) supplemented at the same dosage.

RESULTS

A total amount of 1,335 explants were placed in regeneration, and of these, 279 regenerated shoots were obtained from all varieties, explant types (Fig.3) and media.

Although, significant differences ($p < 0.05$) between the three main factors were noticed (Tab. 2). Hypocotyls on medium 0 were also able to root spontaneously.

Fig. 3 *Direct in vitro shoot organogenesis from hypocotyls /A); cotyledons (B) and leaves (C).*

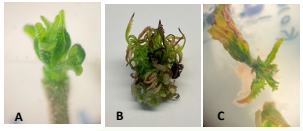


Table 2. *Effect of genotype, type of explant and medium on direct in vitro shoot caulogenesis rate. Count (biological replicates) and number of explants are shown in different columns. Mean of responding explants (%) and significance are presented in the same column. [a]Different letters among the levels of each of the two factors indicate significant differences between them ($p < 0,05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon test. For each factor, mean of responding explants is expressed as a percentage*

Factor	Count	N° Explants	Responding Explants (%)
Variety			
Eletta Campana	98	738	25.0 ± 3.4 [a]
Fibrante	50	332	18.8 ± 3.4 [a]
Felsinea	50	265	16.9 ± 3.6 [a]
Explant			
Hypocotyl	68	448	44.1 ± 4.0 [a]
Cotyledon	68	489	17.0 ± 2.9 [b]
True Leaf	62	398	1.5 ± 0.7 [c]
Medium (mg/L)			
0: No plant regulators	65	438	17.4 ± 3.4[c]
1: TDZ 0.4 + NAA 0.2	42	300	11.3 ± 3.3[c]
2: ZR 2	50	349	27.3 ± 4.6[ab]
3: mT	41	248	31.0 ± 5.0[a]

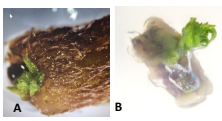
Test 1. No events of direct or indirect caulogenesis were observed. Under the same cultivation conditions (media with M_T), V20 exhibited a greater capacity for callogenesis and a higher competence for regeneration, even if only rhizogenesis was obtained, especially from leaf explants, at the higher NAA dosage. Genotype V13 on TDZ showed lower callus production compared to the media with M_T and no regeneration. These results suggest that genotype V13 may be more recalcitrant to *in vitro* culture.

Test 2. Preliminary results highlighted that caulogenesis from internodal cuttings is feasible using 2,4-D coupled with M_T, TDZ with differences according to the genotype (Tab. 3 and Fig. 4). However, the protocol still needs to be further optimized.

Table 3. *Percentges of regenerations of internodal cuttings on different media. The number of explants tested in each medium is reported in brackets. M.d. = missing data*

Variety	BAP	MT	TDZ	ZTR
V02	m.d	0%	6.7% (30)	0%
V20	2.5% (40)	10% (50)	2% (50)	m.d
V24	0% (50)	5% (20)	6.7% (30)	6.7% (30)

Figure 4. *Caulogenesis from internodal cuttings of V20 on M_T (A) and TDZ (B).*



The best performing Genotype x Tissue x Medium combination was used to verify the efficiency of transformation with *Agrobacterium tumefaciens.* To choose the more efficient strain on *Cannabis* among EHA105 and C58C1 (both carrying the GUS-Int gene) an infiltration test was performed on 5-day shoots (Fig 5).

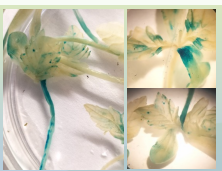


Figure 5. *Blue coloration of stems, leaves and cotyledons of Cannabis shootlets afer EHA105 infiltration*

The EHA105 strain was then used for all the subsequent trials, involving Eletta campana hypocotyls (159) from sterilized seeds. However, we were able to regenerate only 7 shootlets (4.4%), however they were not transformed and appeared white or diaphanous (Fig. 6). More experiments are ongoing in order to optimize the transformation protocol of cannabis hypocotyls.

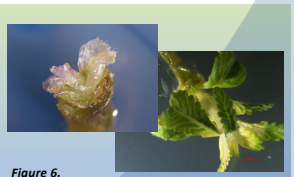


Figure 6. *Diaphanous and white regenerated shootlets*

FINAL CONSIDERATIONS

While regeneration from explants from germinated seeds is easy and does not require the use of specific hormone ratios, regeneration from mature tissues remains of the biggest problems in tissue culture and *Cannabis*. We have achieved several direct regenerations on asexual explants using different hormone ratios and specific hormones; despite the result achieved, the protocol is still under development as we are not yet able to maintain and properly develop the regenerated shoot. The success, at least partial, of the regeneration protocol raises the hope of being able to obtain explants that can be genetically modified by cisgenesis or gene editing.