Industrial Crops & Products 170 (2021) 113691

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

A novel and rapid method for *Agrobacterium*-mediated production of stably transformed *Cannabis sativa* L. plants

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ARTICLE INFO

Keywords:
Cotyledon
Genetic transformation
GUS
Hemp
Hypocotyl
Kanamycin
Meristem
nptII
PCR
Transgenesis
uidA

ABSTRACT

The development of genetically transformed plants is an elusive landmark in *Cannabis sativa* L. breeding. Despite its economic interest, at present, protocols for producing transgenic *C. sativa* plants are scarce. We studied the ability of hypocotyl, cotyledon and meristem explants from six *C. sativa* hemp varieties for transgenic plant regeneration. For this, we firstly evaluated *in vitro* regeneration rates of hypocotyls cultured in medium without plant growth regulators, and cotyledons cultured in medium supplemented with 0.4 mg L^{-1} of thidiazuron (TDZ) and 0.2 mg L^{-1} of α-naphthaleneacetic (NAA). Subsequently, the effect of different kanamycin concentrations (50, 100, 200, 500 and 750 mg L^{-1}) on hypocotyl regeneration rate was determined. Finally, we assessed transformation rates after hypocotyl, cotyledon and meristem co-culture with *Agrobacterium tumefaciens* strain LBA4404 carrying the binary plasmid pBIN19 containing the *β-glucuronidase* (*uidA*) reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) genes. Plant transformation was validated through *in vitro* culture of regenerating shoots in kanamycin-containing selective regeneration medium, by GUS histochemical assay for *uidA* expression, and by PCR amplification of *uidA* and *nptII* genes. Our results showed that hypocotyls reached a higher regeneration rate (53.3 %) than cotyledons (18.1 %) without *Agrobacterium* co-culture. On the other hand, 100 mg L^{-1} kanamycin proved to be the best concentration in terms of regeneration rate (63.3 %) and spontaneous rooting rate of hypocotyl regenerating shoots (12.2 %), which displayed a 7.1 % of albinism rate. After co-culture with *A. tumefaciens* and subsequent culture in antibiotic-containing selective regeneration medium, hypocotyl was the best explant type achieving 23.1 % of regeneration rate, which contrasts with the 1.0 % regeneration rate detected for cotyledons. Transgenic plants were obtained from all explant types evaluated. Although there were significant differences among varieties evaluated, hypocotyls proved to be superior to already-developed meristems, reaching a transformation rate of 5.0 % and 0.8 % respectively. Despite the extremely low regeneration rate of cotyledons after *A. tumefaciens* co-culture, all cotyledon-derived regenerating shoots analyzed were successfully transformed. Our hormone-free protocol doubles the transformation rate of regenerating shoots, also producing transgenic plants three times faster than other already published protocols. This has relevant implications for *C. sativa* breeding, enabling not only genetic transformation, but also the use of new plant breeding techniques such as targeted genome editing by using CRISPR/Cas systems. This may foster the development of *C. sativa* varieties with specific biochemical profiles, or tolerant to biotic and abiotic stresses among others.

1. Introduction

Cannabis sativa L. is a dicotyledonous and angiosperm species with multiple uses belonging to the Cannabaceae family. Cannabinoids are responsible for the pharmacological and psychoactive properties of this species. Since Δ9-tetrahydrocannabinol (THC) was first isolated and characterized by Gaoni and Mechoulam (1964), the therapeutic properties of *C. sativa* have attracted the interest of researchers around the

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https://doi.org/10.1016/j.indcrop.2021.113691
Received 1 March 2021; Received in revised form 25 May 2021; Accepted 27 May 2021
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world. Additionally, hemp (*C. sativa* varieties containing less than 0.3 % w/w of THC) is cultivated for biomass and fiber that constitute feedstock for industrial uses such as energy, construction and automotive markets, and for hempseeds that are components of functional foods and animal feeds (Żuk-Gołaszewska and Gołaszewski, 2020).

Biotechnological approaches such as genetic transformation could be an important landmark in *C. sativa* breeding, as demonstrated in other plant species through the development of improved varieties resistant to biotic and abiotic stresses, with better nutritional and processing qualities, or with increased yields among others (Gosal and Wani, 2018). However, before the implementation of this technique in *C. sativa* species, it is imperative to develop an efficient transformation protocol that allows the regeneration of transgenic plants.

In this respect, there have been some attempts at transformation of *C. sativa*. Successful *Agrobacterium tumefaciens*-mediated transformation of stem and leaf-derived callus suspension cultures from four hemp varieties expressing phosphomannose-isomerase (PMI) gene has been reported (Feeney and Punja, 2003, 2015). Additionally, there are reports of effective establishment of *Agrobacterium rhizogenes*-transformed hairy root cultures showing *β-glucuronidase* (GUS) positive staining from three hemp varieties plus two drug-type varieties (Wahby et al., 2013, 2017). These latter studies also reported *in vivo* and *in vitro* Ri and Ti plasmid-bearing *Agrobacterium* infection of hypocotyl and cotyledonary node explants. *Agrobacterium tumefaciens*-mediated transformation of leaf, male and female flowers, stem, and root tissues from eight hemp varieties using vacuum infiltration has also been achieved, being verified through subsequent detection of GUS and green fluorescence protein (GFP) in the transformed tissues (Deguchi et al., 2020). In the former work, also phytoene desaturase (PDS) gene silencing resulting in an albino phenotype in leaves and male and female flowers was carried out. *Agrobacterium tumefaciens*-mediated transformation of *C. sativa* seedlings from three medical cannabis varieties that transiently expressed GUS gene has also been reported (Sorokin et al., 2020). In addition, nanoparticle-based transient gene transformation of trichomes and leaf cells from one hemp variety, in which transcription of soybean genes and localization of fluorescent-tagged transcription factor proteins were detected, has also been achieved (Ahmed et al., 2020a). Finally, transient transformation with *A. tumefaciens* and Cotton leaf crumple virus (CLCrV) induced gene silencing of PDS and magnesium chelatase subunit I (ChII) genes in leaves from one hemp variety (Schachtsiek et al., 2019), and GFP-transient expression through polyethylene-glycol (PEG)-mediated protoplast transformation have also been obtained (Beard et al., 2021). However, despite the successful genetic transformation of different non-regenerating explants, *C. sativa* recalcitrance to plant regeneration have prevented the recovery of transgenic plants (Feeney and Punja, 2017; Wróbel et al., 2018). It was only recently that regeneration of one *C. sativa* transformed plant has been reported (Zhang et al., 2021).

In this work, we aimed at the development of a novel protocol for the regeneration of stably transformed *C. sativa* plants. For this, we assessed the feasibility of different explants for the production of *Cannabis* transformed plants. Consequently, we compared their transformation rates after co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 containing binary plasmid pBIN19 carrying the *β-glucuronidase* (*uidA*) reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) genes. While the *nptII* gene (Fraley et al., 1983) confers the ability to proliferate on a medium containing normally inhibitory levels of kanamycin in transformed cells, the *uidA* gene (Jefferson et al., 1987) encodes for the *β-glucuronidase* (GUS) enzyme that is commonly used as a reporter gene in GUS histochemical assay for validating the integration and expression of foreign DNA in the transformed cells. In our work, plant transformation was verified through the growth of regenerating shoots on kanamycin-containing selective regeneration medium, evaluation of the *uidA* gene expression by GUS assay in regenerant-derived tissues, and amplification of *uidA* and *nptII* genes by PCR, which is routinely employed for quick detection of cell transformation in plant tissues (Lassner et al., 1989). Due to the lack of studies concerning plant growth inhibitory effects of kanamycin in *in vitro* culture of *C. sativa*, we also performed a dose-response experiment with hypocotyls.

2. Materials and methods

2.1. Materials

2.1.1. Plant material and growth conditions

Seeds from monoecious *C. sativa* short-day varieties 'Ferimon', 'Felina32', 'Fedora17', 'Futura75' and 'USO31', and from dioecious neutral-day variety 'FINOLA' were used in our experiments. Seeds were surface sterilized by manual shaking in 75 % (v/v) ethanol for two min and 30 s, followed by immersion in 30 g L^{-1} of NaClO with 0.1 % (v/v) of Tween 20 for 25 min, and finally washed three times with sterile deionized water. Once sterilized, seeds were germinated in 9 cm diameter plastic Petri dishes containing previously autoclaved semi-solid half-strength MS medium (Murashige and Skoog, 1962) with sucrose (Table 1). Explants were dissected from seven-days-old seedlings grown in plastic Petri dishes with semi-solid germination medium (Table 1) under aseptic conditions. In *C. sativa*, this stage of seedling development is equivalent to the phenological growth stage 11 according to the corresponding BBCH-scale (Mishchenko et al., 2017). Seedlings and explants were grown in a climatic chamber with controlled conditions

Table 1
Media tested for the *in vitro* culture experiments with hypocotyls and cotyledons, and for *A. tumefaciens* LBA4404 infection and co-culture of hypocotyls, cotyledons and already-developed meristems and subsequent *in vitro* culture in selective regeneration media. Media pH was adjusted to 5.8 in all protocol stages (except liquid LB medium in which pH was adjusted to 7.0). Semi-solid medium was employed for all protocol stages except for *Agrobacterium* inoculation. All semi-solid media were supplemented with 3.5 g L^{-1} Gelrite®. **Abbreviations → MS**: Murashige and Skoog medium; **LB**: Luria-Bertani medium; **RIF**: rifampicin; **KAN**: kanamycin; **CTX**: cefotaxime; **AcS**: acetosyringone; **CAR**: Carbenicillin; **TDZ**: thidiazuron; **NAA**: α-naphthaleneacetic acid.

Explant	Protocol stage	Media composition
Seed	Seed germination	½ MS + 1.5 % (w/v) sucrose
Hypocotyl	Control	½ MS + 1.5 % (w/v) sucrose
	Agrobacterium inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5 % (w/v) sucrose + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5 % (w/v) sucrose + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN
Cotyledon	Control	½ MS + 1.5 % (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA
	Agrobacterium inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5 % (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5 % (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN
Meristem	*Agrobacterium* inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5 % (w/v) sucrose + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5 % (w/v) sucrose + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN

(25 ± 1 °C and 60 ± 5% relative humidity) under a 16 h light / 8 h dark photoperiod. The light was provided by Light Emitting Diode (LED) tubes of 18 W and a color temperature of 6000 K, which provided 6, 010 lx and 90.15 μmol m^{-2} s^{-1}. Plants used in this study were grown under license for the cultivation of *C. sativa* for research purposes, issued by the Spanish Ministry of Health *via* the Spanish Agency of Medicines and Health Products (Agencia Española de Medicamentos y Productos Sanitarios or AEMPS) to Ploidy and Genomics Ltd.

2.2. Methods

2.2.1. Hypocotyl and cotyledon explant in vitro culture, and hypocotyl kanamycin dose-response experiments

Cannabis sativa L. plant *in vitro* regeneration efficiency of already published protocols from hypocotyls (Galán-Ávila et al., 2020), and cotyledons (Chaohua et al., 2016) was compared. Regarding kanamycin-resistance of *in vitro* hypocotyl-derived regenerating shoots, a dose-response experiment consisting of adding increasing concentrations (50, 100, 200, 500 and 750 mg L^{-1}) of kanamycin to the semi-solid control medium was performed (Table 1). Culture dishes were examined with an Optika® SZN-6 (OPTIKA S.r.l., Ponteranica, Italy) laboratory stereo zoom microscope equipped with an Optika® C-HP (OPTIKA S.r.l.) digital camera. Explants producing shoots and roots, number of shoots developed on each of responding explants and albino regenerating shoots were counted one month after *in vitro* culture initiation. Media employed as the control for hypocotyl and cotyledon *in vitro* culture experiments are described in Table 1.

2.2.2. Media used, bacterial strain and transformation vector

The Luria-Bertani medium (LB) (Bertani, 1951) was used for all the processes involved in the liquid bacterial culture, always supplemented with the appropriate antibiotic (50 mg L^{-1} of rifampicin and 50 mg L^{-1} of kanamycin). The *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pBIN19 was employed for transformation experiments. The combination strain/plasmid confers the kanamycin resistance for the presence of the *nptII* gene in the plasmid and the rifampicin resistance for the bacterial chromosome. The reporter gene *uidA* present in the pBIN19 binary vector from *A. tumefaciens* strain LBA4404 is interrupted by an intronic sequence to deduce expression only from eukaryotic cells (Bakhsh, 2020).

2.2.3. Agrobacterium tumefaciens co-cultivation of explants and subsequent culture in antibiotic-containing medium

Hypocotyls, cotyledons and the remaining already developed meristems from seven-days-old *C. sativa* seedlings were employed as explants in transformation experiments. The *Agrobacterium* suspension culture was initiated from frozen 25 % glycerol stocks on LB medium preserved at -80 °C. The seeding in 100 mL of fresh LB culture medium containing 50 mg L^{-1} of kanamycin and 50 mg L^{-1} of rifampicin was carried out scratching the surface of the frozen stock with an inoculating loop without thawing, and immediately the tube was dipped in liquid nitrogen to avoid damage in the stock. The bacterial culture was grown for about 24 h at 28 °C under orbital agitation (220 rpm). At optical density at 600 nm (OD600) of ≈1, cells were precipitated by centrifuging at 2236 g for 15 min at RT and resuspended to an OD600 of 0.5 with the sterile *Agrobacterium* inoculation medium (Table 1). Then, the explants were placed in Petri dishes with the prepared medium for about 40 min for static infection. Immediately following the inoculation, the explants were cultured in Petri dishes with semi-solid co-culture medium (Table 1) for 4 days in the growth conditions indicated above. Subsequently, the explants were cultured in the semi-solid selective regeneration medium (Table 1) for approximately 26 days in equal conditions.

Culture dishes were examined with an Optika® SZN-6 laboratory stereo zoom microscope equipped with an Optika® C-HP digital camera. Explants producing shoots and roots, number of shoots developed on each of responding explants and albino regenerating shoots were counted one month after *in vitro* culture initiation. Media used in the different *C. sativa* transformation protocol stages are described in Table 1.

2.2.4. Visual analysis of GUS expression in hypocotyls, cotyledons and already-developed meristems after regenerant-derived tissue incubation with X-Gluc

Approximately one month after *in vitro* culture of explants, samples (leaves or newly developed meristems) were taken from the top of regenerated plants, incubated with X-Gluc (0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 100 mM $NaPO_4$ pH 7, 10.0 mM EDTA, 0.1 % Triton X-100, 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at 37 °C for approximately 12 h as described by Jefferson (1987), and decolored through a graded ethanol series in deionized water (75 % EtOH for 2 h + 90 % EtOH for 2 h + 100 % EtOH o/night). High-resolution images of the different events observed were recorded with an Optika® SZN-6 (OPTIKA S.r.l.) laboratory stereo zoom microscope equipped with an Optika® C-HP (OPTIKA S.r.l.) digital camera.

2.2.5. Primer design and polymerase chain reaction (PCR) detection of β-glucuronidase (uidA) and kanamycin resistance neomycin phosphotransferase II (nptII) genes

Approximately one month after explant *in vitro* culture initiation, samples (leaves or newly developed meristems) were taken from the top of regenerated plants and genomic DNA was extracted following the SILEX method (Vilanova et al., 2020). DNA quality and integrity were checked by agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Integration of the T-DNA in the plant genome was confirmed by PCR amplifying a 206-bp fragment of the *uidA* reporter gene (Forward primer 5'-CCCATCATGACCTTGCCAAG-3', Reverse primer 5'-CAGGGCTACAAAATCACGGG-3') and a 795-bp fragment of the *nptII* gene (Forward primer 5'-ATGATTGAACAAGATGGATTGCAC-3', Reverse primer 5'-TCAGAAGAACTCGTCAAGAAGGCG-3'). PCR amplifications were performed in a volume of 25 μL including 19 μL water, 2.5 μL 10 × PCR buffer, 1 μL $MgCl_2$ 50 mM, 0.5 μL dNTPs 10 mM each, 0.5 μL of each primer 10 mM, 0.5 μL Taq DNA Polymerase (1 U/μL), and 0.5 μL DNA template 100 ng/μL. The PCR program used was the following: 94 °C for 5 min for DNA denaturation, 30 cycles of 20 s at 94 °C followed by 30 s at 60 °C (either for *uidA* and *nptII* genes), and of 20 s at 72 °C and finally 72 °C for 1 min for the last step of extension. Only regenerating shoots that concurrently showed a green phenotype after culture on selective regeneration medium, uniform expression (non-chimeric) of the *uidA* gene after X-Gluc regenerant-derived tissue incubation by GUS histochemical assay, plus amplification of the *uidA* and *nptII* genes by PCR, were considered as transformed regenerating shoots.

2.2.6. Data analyses

Depending on the experiment analyzed, the number of shoots per responding explant and plant regeneration, spontaneous rooting, albinism and/or transformation rates were compared for the different factors evaluated (explant type, hemp variety and/or kanamycin concentration). Experiments were repeated at least three times. Each replicate consisted of a Petri dish containing at least 5 explants coming from 5 different seedlings per variety in the case of hypocotyls, and at least 10 explants coming from 5 different seedlings per variety in the case of cotyledons. Independence among variables (Durbin-Watson test), homoscedasticity (Bartlett's test for mean variance analysis or Fligner-Killeen median test), and normality (Shapiro-Wilk test) were evaluated for the data. Subsequently, Kruskal-Wallis non-parametric tests followed by pairwise Wilcoxon or Nemenyi tests ($p < 0.05$) were used to statistically determine significant differences between levels of each factor evaluated. Pairwise testing was based on whether analysis comprised more than one pairwise comparison (Nemenyi) or not (Wilcoxon). Statistical analysis was carried out using R software (R Core

Team, 2019).

3. Results

3.1. Efficiency comparison of C. sativa hypocotyl and cotyledon in vitro plant regeneration protocols

We compared the ability of *C. sativa* hypocotyls and cotyledons for plant *in vitro* regeneration without *Agrobacterium* co-culture. The different regeneration patterns observed for hypocotyls and cotyledons are illustrated in Figs. 1 and 2 respectively. While hypocotyls developed at most one (Fig. 1A) or two shoots per explant (Fig. 1B), they were able to develop roots spontaneously (Fig. 1C). In contrast, single-shoot regeneration (Fig. 2A and B), and multiple-shoot regeneration (Fig. 2C and D) were observed in cotyledons, although despite the vigorous growth of cotyledon-derived regenerating shoots (Fig. 2E), they were incapable of developing roots spontaneously.

Furthermore, hypocotyls had a significant higher regeneration rate

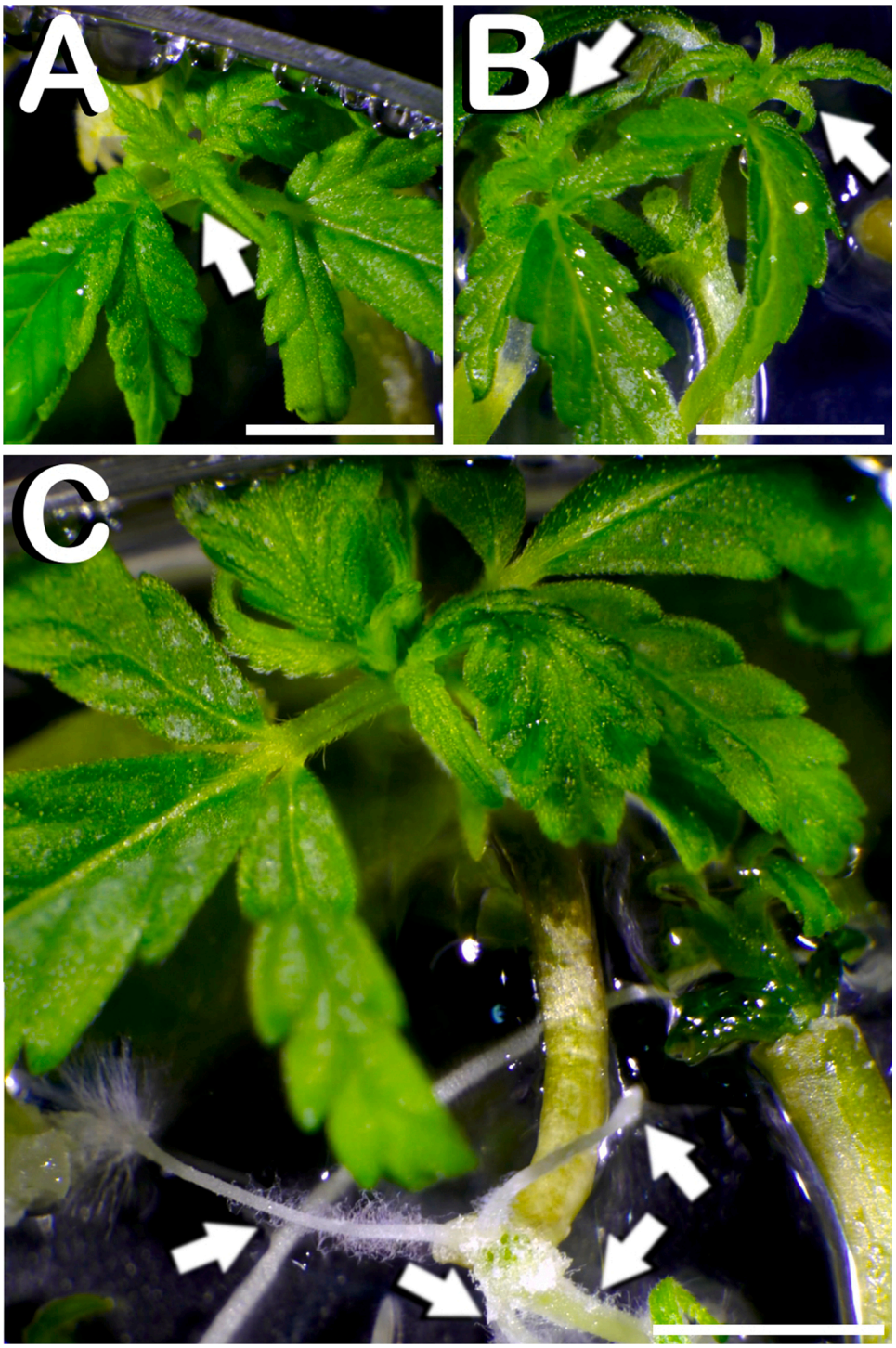


Fig. 1. Different *C. sativa* hypocotyl-derived plant regeneration patterns. The different images are described as follows: (A) Single-shoot hypocotyl regeneration three weeks after *in vitro* culture initiation: arrow points to shoot. (B) Regeneration of two shoots from a hypocotyl three weeks after explant *in vitro* culture: arrows point to both shoots. (C) Spontaneous rooting of hypocotyl derived regenerating shoots after three weeks of *in vitro* culture: arrows point to roots. Scale bars (A–C): 4 mm.

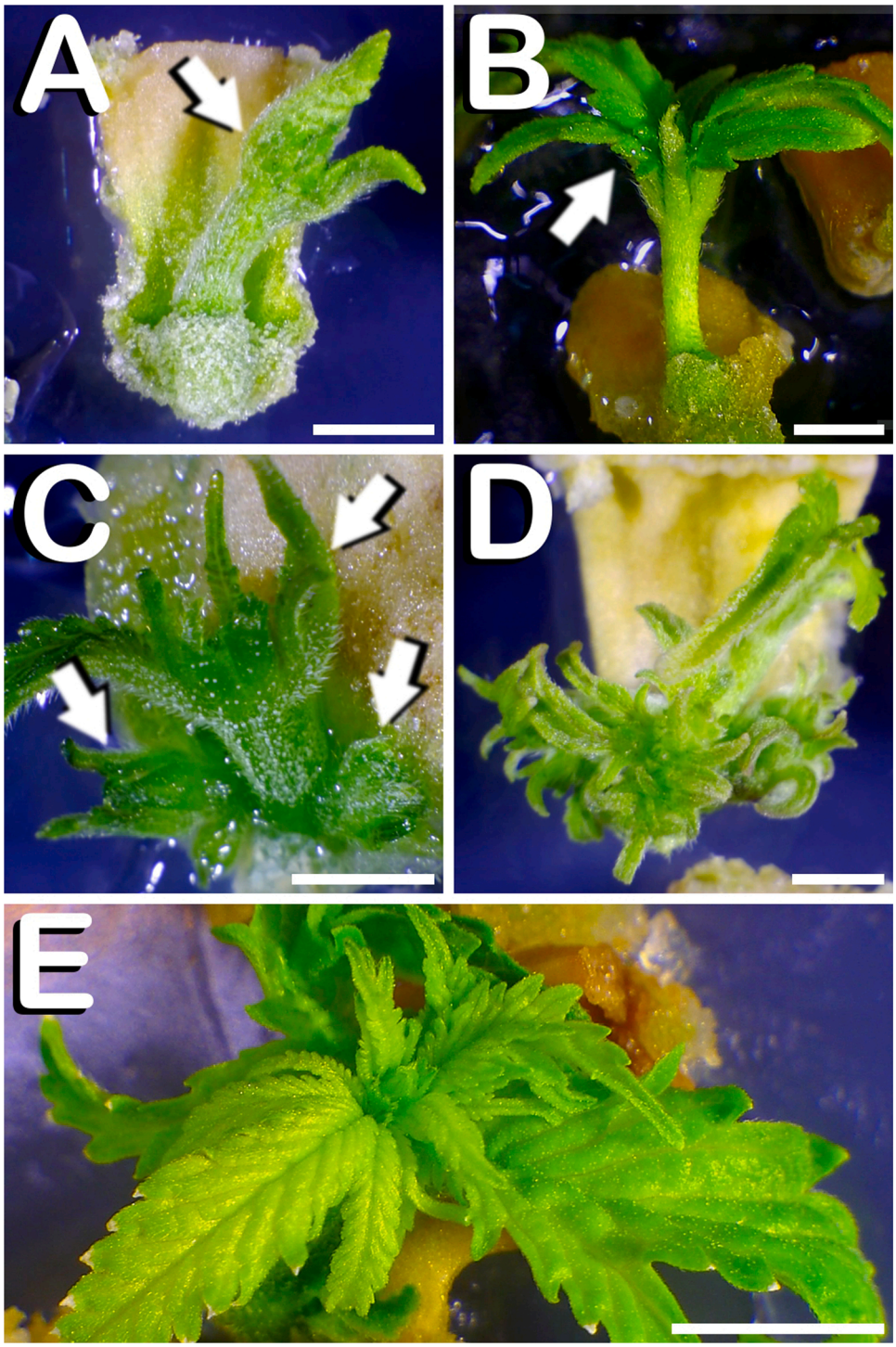


Fig. 2. Different *C. sativa* cotyledon-derived plant regeneration patterns. The different images are described as follows: (A) Single-shoot cotyledon regeneration one week after *in vitro* culture initiation: arrow points to shoot. (B) Cotyledon-derived regenerating shoot two weeks after *in vitro* culture: arrow points to shoot. (C) Regeneration of three shoots from a cotyledon one week after explant *in vitro* culture: arrows point to shoots. (D) Multiple shoot regeneration from a single cotyledon after two weeks of *in vitro* culture. (E) Shoot development one month after cotyledon *in vitro* culture initiation. Scale bars (A-D): 2 mm. Scale bar (E): 4 mm.

(53.3 %) than cotyledons (18.1 %) (Table 2). Significant differences were also detected among both types of explant in terms of spontaneous rooting rate. Almost 26 % of hypocotyl-derived regenerating shoots developed roots (Table 2), in contrast with cotyledon-derived regenerating shoots, which were unable to develop roots in the evaluated medium. On the other hand, cotyledon-derived regenerating shoots developed more shoots per responding explant compared to hypocotyl-derived regenerating shoots. Both explant types reached 1.6 and 1.3 shoots respectively (Table 2). It should be noted that neither cotyledons nor hypocotyls developed albino plants after *in vitro* culture in control media (Table 2).

Significant differences were detected among the different varieties studied indicating that there is a genotype effect on hypocotyl-derived plant regeneration. While 'Fedora17' had the highest regeneration rate of this experiment with 76.5 % of responding explants (Table 3), 'FINOLA' displayed the lowest hypocotyl plant regeneration with 36.4 % (Table 3). Additionally, some varieties had a significantly higher capacity for spontaneous rooting than others. Again, while 'Fedora17' produced the best results of the experiment, 'FINOLA' yielded the lower percentages of regeneration. These varieties achieved 47.0 % and 13.6 % of spontaneous rooting rate respectively (Table 3). The genotype had no effect on cotyledon-derived plant regeneration and no significant

Table 2
Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl and cotyledon-derived regenerating shoots after *in vitro* control, kanamycin and transformation treatments. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Explant	Treatment	Responding explants (%)	Shoots/responding explant	Spontaneous rooting (%)	Albino regenerating shoots (%)
Hypocotyl	Control	$53.3^{a} \pm 4.3$	$1.3^{a} \pm 0.1$	$25.9^{a} \pm 3.8$	$0.0^{a} \pm 0.0$
Cotyledon	Control	$18.1^{b} \pm 2.3$	$1.6^{a} \pm 0.1$	$0.0^{b} \pm 0.0$	$0.0^{a} \pm 0.0$
Hypocotyl	0 mg L^{-1} kanamycin	$53.3^{ab} \pm 4.3$	$1.3^{a} \pm 0.1$	$25.9^{a} \pm 3.8$	$0.0^{b} \pm 0.0$
	50 mg L^{-1} kanamycin	$60.0^{ab} \pm 7.0$	$1.3^{a} \pm 0.1$	$4.0^{bc} \pm 2.8$	$0.0^{b} \pm 0.0$
	100 mg L^{-1} kanamycin	$63.3^{a} \pm 7.0$	$1.7^{a} \pm 0.1$	$12.2^{b} \pm 4.7$	$7.1^{b} \pm 5.0$
	200 mg L^{-1} kanamycin	$64.0^{a} \pm 6.9$	$1.7^{a} \pm 0.1$	$6.0^{bc} \pm 3.4$	$12.5^{b} \pm 5.9$
	500 mg L^{-1} kanamycin	$56.9^{ab} \pm 7.0$	$1.3^{a} \pm 0.1$	$0.0^{c} \pm 0.0$	$90.9^{a} \pm 6.3$
	750 mg L^{-1} kanamycin	$40.8^{b} \pm 7.1$	$1.2^{a} \pm 0.1$	$0.0^{c} \pm 0.0$	$93.3^{a} \pm 6.7$
Hypocotyl	Co-culture + selective regeneration	$23.1^{a} \pm 1.3$	$1.2^{a} \pm 0.0$	$2.1^{a} \pm 0.5$	$18.0^{a} \pm 2.6$
Cotyledon	Co-culture + selective regeneration	$1.0^{b} \pm 0.2$	$1.0^{a} \pm 0.0$	$0.0^{b} \pm 0.0$	$16.7^{a} \pm 9.0$
Hypocotyl	Control	$53.3^{a} \pm 4.3$	$1.3^{a} \pm 0.1$	$25.9^{a} \pm 3.8$	$0.0^{b} \pm 0.0$
	Co-culture + selective regeneration	$23.1^{b} \pm 1.3$	$1.2^{b} \pm 0.0$	$2.1^{b} \pm 0.5$	$18.0^{a} \pm 2.6$
Cotyledon	Control	$18.1^{a} \pm 2.3$	$1.6^{a} \pm 0.1$	$0.0^{a} \pm 0.0$	$0.0^{b} \pm 0.0$
	Co-culture + selective regeneration	$1.0^{b} \pm 0.2$	$1.0^{b} \pm 0.0$	$0.0^{a} \pm 0.0$	$16.7^{a} \pm 9.0$

Different letters among the levels of each factor indicate significant differences between them ($p < 0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

Table 3
Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl and cotyledon-derived regenerating shoots from different varieties after *in vitro* culturing in their respective control media. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Explant	Variety	Responding explants (%)	Shoots/responding explant	Spontaneous rooting (%)	Albino regenerating shoots (%)
Hypocotyl	'Fedora17'	$76.5^{a} \pm 7.4$	$1.3^{a} \pm 0.1$	$47.1^{a} \pm 8.7$	$0.0^{a} \pm 0.0$
	'Felina32'	$39.1^{b} \pm 10.4$	$1.2^{a} \pm 0.1$	$17.4^{b} \pm 8.1$	$0.0^{a} \pm 0.0$
	'Ferimon'	$51.8^{ab} \pm 9.8$	$1.2^{a} \pm 0.1$	$25.9^{ab} \pm 8.6$	$0.0^{a} \pm 0.0$
	'FINOLA'	$36.4^{b} \pm 10.5$	$1.6^{a} \pm 0.3$	$13.6^{b} \pm 7.5$	$0.0^{a} \pm 0.0$
	'Futura75'	$54.5^{ab} \pm 15.7$	$1.0^{a} \pm 0.0$	$18.2^{b} \pm 12.2$	$0.0^{a} \pm 0.0$
	'USO31'	$50.0^{ab} \pm 12.1$	$1.6^{a} \pm 0.2$	$16.7^{b} \pm 9.0$	$0.0^{a} \pm 0.0$
Cotyledon	'Fedora17'	$20.2^{a} \pm 4.4$	$1.5^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Felina32'	$11.8^{a} \pm 5.6$	$1.2^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Ferimon'	$17.3^{a} \pm 4.2$	$1.7^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm \pm \pm 0.0$
	'FINOLA'	$26.3^{a} \pm 7.2$	$2.0^{a} \pm 0.4$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Futura75'	$4.5^{a} \pm 4.5$	$1.0^{*} \pm *$	$0.0^{a} \pm 0.0$	$0.0^{*} \pm *$
	'USO31'	$21.4^{a} \pm 7.9$	$1.2^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$

Different letters among the levels of each factor indicate significant differences between them ($p < 0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

* As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

differences were detected among the different parameters evaluated (Table 3).

3.2. Effect of kanamycin on C. sativa hypocotyl regeneration in in vitro culture

Different parameters were compared after *in vitro* hypocotyl culture in the control medium and after the addition of increasing kanamycin concentrations. Shoot patterns developed during hypocotyl *in vitro* culture in kanamycin containing media can be observed in Fig. 3. While some explants produced synchronous development of albino regenerating shoots (Fig. 3A and B), other hypocotyl-derived regenerating shoots, even coming from the same explant, showed different ability for kanamycin resistance (Fig. 3C and D). Although hypocotyl-derived regenerating shoots cultured in kanamycin containing medium displayed an albino phenotype, they were able to exhibit a vigorous growth one month after *in vitro* culture initiation (Fig. 3E and F).

A decrease in regeneration and spontaneous rooting rates was observed with increasing kanamycin concentrations. Specifically, the highest kanamycin concentration tested (750 mg L^{-1}) decreased the regeneration rate to an average of 40.8 % (Table 2). Conversely, compared with the control, 100 mg L^{-1} and 200 mg L^{-1} slightly increased the hypocotyl plant regeneration rate, attaining 63.3 % and 64.0 % of responding explants respectively (Table 2). The best result for spontaneous rooting rate in kanamycin-containing medium (12.2 %), was registered for the concentration of 100 mg L^{-1} (Table 2). Although this concentration caused a significant decrease of the rooting rate with respect to the control treatment (Table 2), it showed the best shoot regeneration and rooting rates on kanamycin, which led us to choose it for the subsequent transformation experiments. Concentrations ranging from 500 mg L^{-1} to 750 mg L^{-1} prevented spontaneous root formation in regenerating shoots (Table 2). On the other hand, an increase in the albinism rate was observed as kanamycin concentrations raised. While no albino plants were detected in 50 mg L^{-1} of kanamycin concentration, albino plants were already observed at 100 mg L^{-1}, with a 7.1 % of albinism rate (Table 2). The higher kanamycin concentrations tested in our work (500 mg L^{-1} and 750 mg L^{-1}), resulted, respectively, in 90.9 % and 93.3 % of albino plants (Table 2). Apart from the control, only one of the evaluated treatments showed a significant influence of the genotype in some of the parameters evaluated. Specifically, in the 100 mg L^{-1} kanamycin concentration treatment, regeneration rate and the number of shoots per responding explant significantly differed among *C. sativa* varieties evaluated (Table 4). 'Felina32' (83.3 %) and 'Fedora17' (77.8 %) exhibited the best plant regeneration rate, contrasting with

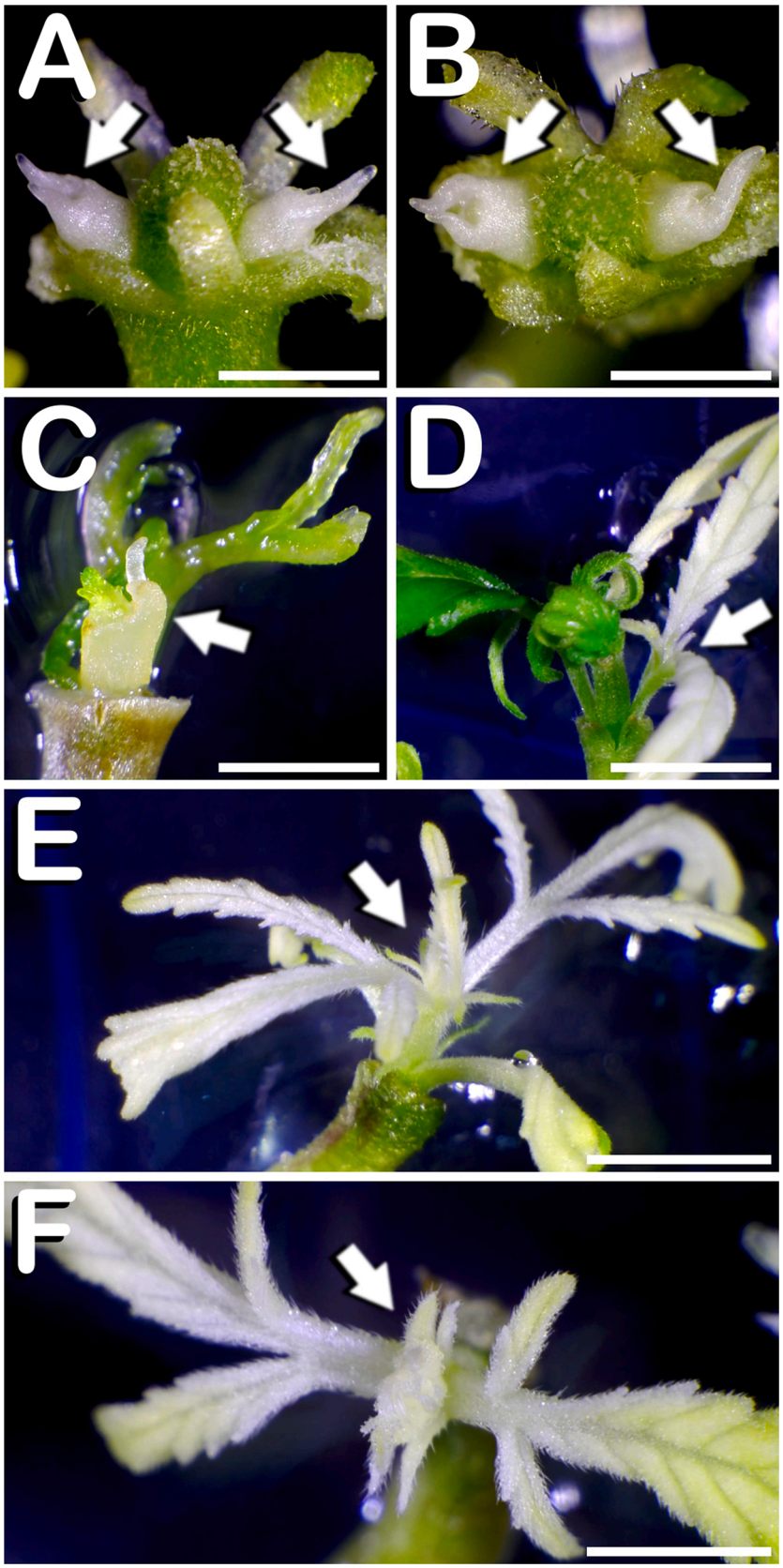


Fig. 3. Shoot development patterns observed during hypocotyl *in vitro* culture in different kanamycin concentrations containing media. The different images are described as follows: (A) Albino hypocotyl-derived regenerating shoots developed in medium with 200 mg L^{-1} kanamycin four days after *in vitro* culture initiation (front view): arrows point to both primordia. (B) Albino hypocotyl-derived regenerating shoots developed in medium with 200 mg L^{-1} kanamycin four days after *in vitro* culture initiation (top view): arrows point to both primordia. (C) Kanamycin-resistant and non-resistant regenerating shoots arising from the top of a *Cannabis* hypocotyl one week after *in vitro* culture in medium containing 100 mg L^{-1} kanamycin: arrow points to kanamycin non-resistant shoot. (D) Kanamycin-resistant and non-resistant hypocotyl-derived regenerating shoot developed in medium with 100 mg L^{-1} kanamycin two weeks after *in vitro* culture: arrow points to kanamycin non-resistant shoot. (E) One-month-old hypocotyl-derived albino shoot (front view) developed in medium with 500 mg L^{-1} kanamycin: arrow points to shoot primordia. (F) One-month-old hypocotyl-derived albino shoot (top view) developed in medium with 500 mg L^{-1} kanamycin: arrow points to shoot primordia. Scale bars (A, B): 1.31 mm; Scale bar (C): 2.64 mm; Scale bars (D-F): 4 mm.

Table 4
Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl-derived regenerating shoots from different varieties after *in vitro* culturing in control medium supplemented with different kanamycin concentrations. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Treatment	Variety	Responding explants (%)	Shoots/responding explant	Spontaneous rooting (%)	Albino regenerating shoots (%)
50 mg L^{-1} kanamycin	'Fedora17'	$77.8^{a} \pm 14.7$	$1.8^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Felina32'	$28.6^{a} \pm 18.4$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Ferimon'	$66.7^{a} \pm 16.7$	$1.5^{a} \pm 0.3$	$11.1^{a} \pm 11.1$	$0.0^{a} \pm 0.0$
	'FINOLA'	$55.6^{a} \pm 17.6$	$1.2^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Futura75'	$66.7^{a} \pm 21.0$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'USO31'	$60.0^{a} \pm 16.3$	$1.2^{a} \pm 0.2$	$10.0^{a} \pm 10.0$	$0.0^{a} \pm 0.0$
100 mg L^{-1} kanamycin	'Fedora17'	$77.8^{a} \pm 14.7$	$1.4^{ab} \pm 0.2$	$11.1^{a} \pm 11.1$	$0.0^{a} \pm 0.0$
	'Felina32'	$83.3^{a} \pm 16.7$	$1.0^{b} \pm 0.0$	$0.0^{a} \pm 0.0$	$20.0^{a} \pm 20.0$
	'Ferimon'	$66.7^{ab} \pm 16.7$	$1.7^{ab} \pm 0.2$	$22.2^{a} \pm 14.7$	$16.7^{a} \pm 16.7$
	'FINOLA'	$66.7^{ab} \pm 16.7$	$2.5^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Futura75'	$16.7^{b} \pm 16.7$	$2.0^{*} \pm *$	$0.0^{a} \pm 0.0$	$0.0^{*} \pm *$
	'USO31'	$60.0^{ab} \pm 16.3$	$2.0^{ab} \pm 0.4$	$30.0^{a} \pm 15.3$	$0.0^{a} \pm 0.0$
200 mg L^{-1} kanamycin	'Fedora17'	$40.0^{a} \pm 16.3$	$1.5^{a} \pm 0.3$	$10.0^{a} \pm 10.0$	$25.0^{a} \pm 25.0$
	'Felina32'	$66.7^{a} \pm 21.1$	$1.5^{a} \pm 0.3$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Ferimon'	$66.7^{a} \pm 16.7$	$2.0^{a} \pm 0.4$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'FINOLA'	$55.6^{a} \pm 17.6$	$1.6^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Futura75'	$83.3^{a} \pm 16.7$	$2.0^{a} \pm 0.3$	$0.0^{a} \pm 0.0$	$40.0^{a} \pm 24.5$
	'USO31'	$80.0^{a} \pm 13.3$	$1.6^{a} \pm 0.3$	$20.0^{a} \pm 13.3$	$12.5^{a} \pm 12.5$
500 mg L^{-1} kanamycin	'Fedora17'	$50.0^{a} \pm 16.7$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'Felina32'	$33.3^{a} \pm 21.1$	$2.0^{a} \pm 1.0$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'Ferimon'	$55.6^{a} \pm 17.6$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$50.0^{a} \pm 28.9$
	'FINOLA'	$60.0^{a} \pm 16.3$	$1.5^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'Futura75'	$50.0^{a} \pm 22.4$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$50.0^{a} \pm 50.0$
	'USO31'	$80.0^{a} \pm 13.3$	$1.2^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
750 mg L^{-1} kanamycin	'Fedora17'	$20.0^{a} \pm 13,3$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'Felina32'	$50.0^{a} \pm 22,4$	$1.3^{a} \pm 0.3$	$0.0^{a} \pm 0.0$	$66.7^{a} \pm 33.3$
	'Ferimon'	$33.3^{a} \pm 16,7$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'FINOLA'	$62.5^{a} \pm 18,3$	$1.4^{a} \pm 0.2$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'Futura75'	$33.3^{a} \pm 21,1$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$100.0^{a} \pm 0.0$
	'USO31'	$50.0^{a} \pm 16,7$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$75.0^{a} \pm 25.0$

Different letters among the levels of each factor indicate significant differences between them ($p < 0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.
* As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

'Futura75' which was the worst variety with 16.7 % of explants developing shoots (Table 4). 'FINOLA' achieved the highest number of shoots per responding explant with 2.5 shoots, while 'Felina32' only produced 1.0 shoot per responding hypocotyl (Table 4).

3.3. Effect of A. tumefaciens co-culture and subsequent explant culture in antibiotic-containing selective regeneration medium in in vitro C. sativa regeneration, spontaneous rooting and albinism rates

Agrobacterium co-culture and successive explant culture in antibiotic-containing selective regeneration medium reduced *in vitro* plant regeneration ability of hypocotyls and cotyledons, although once again, hypocotyl explants displayed a superior efficiency (23.1 %) than cotyledons (1.0 %) under these experimental conditions (Table 2). Both explant types significantly diminished their respective regeneration rates in comparison with control treatment, from 53.3%–23.1% in the case of hypocotyls, and from 18.1 % to 1.0 % in cotyledons (Table 2). Although there were no significant differences among both types of explant in terms of the number of shoots per responding explant, when explants were analyzed separately, this parameter was significantly reduced in comparison with control treatments. While hypocotyl decreased from 1.3 shoots per responding explant under control conditions to 1.2 shoots after culture with *Agrobacterium* and antibiotics, this same trend was observed in cotyledons, which reduced from 1.6 to 1.0 shoot per responding explant (Table 2). Regarding spontaneous rooting of hypocotyl-derived regenerating shoots, both *Agrobacterium* and antibiotics explant exposure promoted a significant rate decrease which ranged from 25.9 % in the control treatment to 2.1 % after *Agrobacterium* and antibiotic exposure (Table 2). No significant differences were detected among hypocotyl and cotyledon explants in terms of albinism rate, which reached, respectively, 18.0 % and 16.7 % of albino regenerating shoots (Table 2). The only parameter significantly influenced by the genotype was the albinism rate of hypocotyl-derived regenerating shoots, varying from 45.4 % for 'Futura75' regenerating shoots, to 4.2 % for 'USO31' regenerated plants (Table 5).

3.4. Effect of explant on the production of C. sativa transgenic plants after A. tumefaciens co-culture and subsequent explant culture in antibiotic-containing selective regeneration medium

Transgenic plants were obtained from all explants evaluated, although hypocotyls, cotyledons and already-developed meristems presented different transformation rates (Table 6). In total, nine transgenic plants were obtained from all the experiments: six transformed plants from hypocotyls (four plants from 'Futura75', one plant from 'Ferimon', and one plant from 'USO31'), two transformed plants from cotyledons (both plants from 'USO31' variety), and one transformed plant from an already-developed meristem (from 'USO31'). Hypocotyls achieved a higher transformation rate than already-developed meristems. While 5.0 % of hypocotyl-derived regenerating shoots were successfully transformed (Table 6), only 0.8 % of already developed meristems developed a transformed regenerating shoot (Table 6). As only two cotyledon-derived regenerating shoots grew enough to be analyzed, the cotyledon transformation rate was not statistically compared with the transformation rates of the remaining explants. However, both cotyledon-derived regenerating shoots were stably transformed (Table 6).

Furthermore, as only two transgenic plants from cotyledons, and one

Table 5
Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* cotyledon and hypocotyl-derived regenerating shoots from different varieties after co-culture with *Agrobacterium tumefaciens* LBA4404 containing binary plasmid pBIN19 carrying *uidA* reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) gene, and culture in their respective selective regeneration media. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Explant	Variety	Responding explants (%)	Shoots/explant	Spontaneous rooting (%)	Albino regenerating shoots (%)
Hypocotyl	'Fedora17'	$18.2^{a} \pm 3.3$	$1.2^{a} \pm 0.1$	$2.9^{a} \pm 1.4$	$8.0^{ab} \pm 5.5$
	'Felina32'	$20.3^{a} \pm 3.0$	$1.1^{a} \pm 0.0$	$4.5^{a} \pm 1.6$	$19.4^{ab} \pm 6.7$
	'Ferimon'	$21.8^{a} \pm 3.2$	$1.1^{a} \pm 0.1$	$1.8^{a} \pm 1.0$	$20.6^{ab} \pm 7.0$
	'FINOLA'	$24.4^{a} \pm 3.3$	$1.2^{a} \pm 0.1$	$1.2^{a} \pm 0.8$	$14.6^{ab} \pm 5.6$
	'Futura75'	$29.1^{a} \pm 3.8$	$1.0^{a} \pm 0.0$	$0.7^{a} \pm 0.7$	$45.4^{a} \pm 8.8$
	'USO31'	$24.9^{a} \pm 3.1$	$1.3^{a} \pm 0.1$	$1.5^{a} \pm 0.9$	$4.2^{b} \pm 2.9$
Cotyledon	'Fedora17'	$0.7^{a} \pm 0.5$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	'Felina32'	$0.3^{a} \pm 0.3$	1.0* ± *	$0.0^{a} \pm 0.0$	0.0* ± *
	'Ferimon'	$0.3^{a} \pm 0.3$	1.0* ± *	$0.0^{a} \pm 0.0$	0.0* ± *
	'FINOLA'	$0.3^{a} \pm 0.3$	1.0* ± *	$0.0^{a} \pm 0.0$	100.0* ± *
	'Futura75'	$0.4^{a} \pm 0.4$	1.0* ± *	$0.0^{a} \pm 0.0$	0.0* ± *
	'USO31'	$3.2^{a} \pm 0.9$	$1.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$16.7^{a} \pm 11.2$

Different letters among the levels of each factor indicate significant differences between them ($p < 0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.
* As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

Table 6
Effect of explant on the production of *Cannabis sativa* L. transgenic plants after co-culture of hypocotyls, cotyledons and already-developed meristems with *Agrobacterium tumefaciens* LBA4404 containing binary plasmid pBIN19 carrying *uidA* reporter gene and the kanamycin resistance neomycin phosphotransferase (*nptII*) gene, and subsequent explant culture in their respective selective regeneration media. Only were considered transformed regenerating shoots those that simultaneously exhibited a green phenotype after culture on selective regeneration medium, and uniform expression (non-chimeric) of the *uidA* gene after X-Gluc regenerant-derived tissue incubation by GUS histochemical assay, plus amplification of the *uidA* and *nptII* genes by PCR.

Explant	Variety	Transformation rate (%)	n
Hypocotyl	Pooled varieties	$5.0^{a} \pm 2.0$	120
Meristem	Pooled varieties	$0.8^{b} \pm 0.8$	130
Cotyledon	Pooled varieties	100.0* ± 0.0	2
Hypocotyl	'Fedora17'	$0.0^{b} \pm 0.0$	16
	'Felina32'	$0.0^{b} \pm 0.0$	22
	'Ferimon'	$5.9^{ab} \pm 5.9$	17
	'FINOLA'	$0.0^{b} \pm 0.0$	19
	'Futura75'	$28.6^{a} \pm 12.5$	14
	'USO31'	$3.1^{b} \pm 3.1$	32

Different letters among the levels of each factor indicate significant differences between them ($p < 0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.
* Not analyzed statistically.

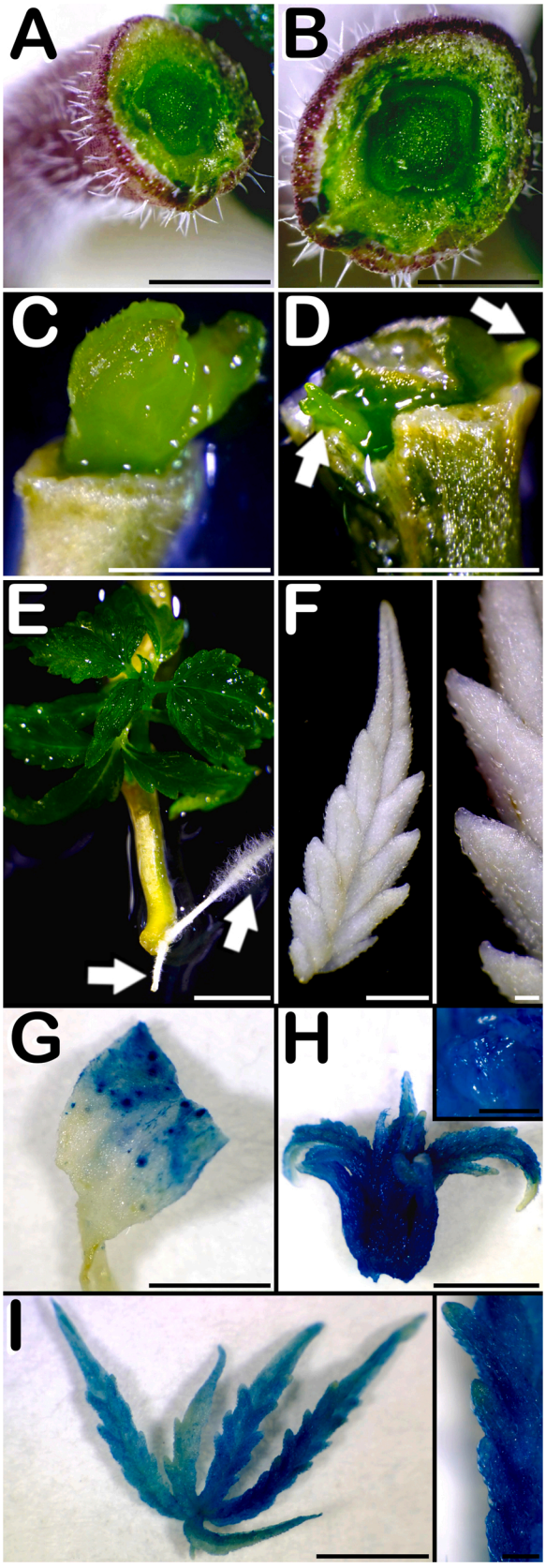
transformed plant from an already-developed meristem were obtained, the genotype effect on transgenic plant regeneration was only evaluated for hypocotyl explants. Significant differences were detected among varieties studied in terms of the transformation rate of hypocotyl-derived regenerating shoots. 'Futura75' stood out significantly among the rest of the evaluated varieties, reaching 28.6 % of hypocotyl-derived transformed regenerating shoots (Table 6), followed by 'Ferimon' and 'USO31' with, respectively, 5.9 % and 3.1 % of transformation rate (Table 6). No transgenic plants were obtained from 'Fedora17', 'Felina32' and 'FINOLA' hypocotyls (Table 6).

3.5. *uidA and nptII gene expression in C. sativa in vitro regenerating shoots*

Hypocotyls dissected from seven-days-old *C. sativa* seedlings (Fig. 4A and B) were co-cultivated with *A. tumefaciens* LBA4404 for 4 days. During co-cultivation, both single hypocotyl-derived regeneration (Fig. 4C), and synchronized regeneration of various primordia in the periphery of the organ (arrows in Fig. 4D) were observed. After the co-cultivation period, explants were transferred to a selective regeneration medium. Within 16 days after initiation of the hypocotyl *in vitro* culture, we were able to observe the spontaneous rooting of *Cannabis* regenerating shoots developed on selective regeneration medium (Fig. 4E). Approximately one month after *in vitro* culture initiation, samples from regenerating shoots were incubated in X-Gluc and decolored. Leaves coming from non-transformed regenerating shoots were white (Fig. 4F), with no signals of *uidA* gene expression (insert in Fig. 4F). Conversely, *Cannabis* leaves from one-month-old hypocotyl-derived transformed shoots showed different GUS staining patterns. On the one hand, some leaf samples showed non-uniform *uidA* expression (Fig. 4G), being characterized by blue spots with different color intensity irregularly distributed along the putatively chimeric tissue. Furthermore, newly-formed primordia derived from transformed shoots showed strong and uniform GUS staining (Fig. 4H). Even the whole shoot apical meristem (SAM) acquired an intense dark-blue coloration (insert in Fig. 4H). Finally, also some leaf samples coming from one-month-old hypocotyl-derived transformed shoots showed strong and uniform GUS staining (Fig. 4I), which also reached the leaflet outline (insert in Fig. 4I).

Regarding cotyledon-derived regenerant genetic transformation, explants dissected from seven-days-old *C. sativa* seedlings (Fig. 5A) were co-cultured with *A. tumefaciens* LBA4404 during 4 days. During co-culture, single cotyledon-derived regenerating shoots (Fig. 5B) were observed. After co-culture, explants were transferred to a selective regeneration medium. After 14 days of *in vitro* culture of cotyledons, regenerating shoots reached ~1 cm in length (Fig. 5C). Approximately one month after *in vitro* culture initiation, shoot samples were incubated in X-Gluc and decolored. While leaf samples coming from non-transformed regenerating shoots were white, with no signals of *uidA* expression, some leaf samples from one-month-old cotyledon-derived transformed regenerating shoots displayed strong and uniform GUS staining, acquiring an intense dark-blue coloration, although the tip of some leaflets did not show any signal of GUS staining (Fig. 5D and E).

Concerning meristem transformation, already-developed meristems dissected from seven-days-old *C. sativa* seedlings (Fig. 6A), which contained the whole shoot apical meristem (SAM) (Fig. 6B), were co-cultured with *A. tumefaciens* LBA4404 for 4 days. After that time, explants were transferred to a selective regeneration medium. Approximately one month after *in vitro* culture initiation, leaf samples were taken from regenerating shoots, which showed a prominent growth (Fig. 6C), incubated with X-Gluc and decolored. While no evidence of GUS staining was detected in samples coming from non-transgenic



(caption on next column)

Fig. 4. Genetic transformation of *C. sativa* hypocotyl-derived *in vitro* regenerating shoots and GUS staining. The different images are described as follows: (A) Newly dissected hypocotyl from a seven-days-old hemp seedling. (B) Detail of the transversal section of a newly dissected hypocotyl just before co-cultivation with *Agrobacterium*. (C) Shoot *in vitro* regeneration from *Cannabis* hypocotyl after three-day co-culture with *Agrobacterium*. (D) Two primordia arising from the top of a *Cannabis* hypocotyl after three-day co-culture with *Agrobacterium*: arrows point to both primordia. (E) Spontaneously-rooted *Cannabis* hypocotyl-derived regenerating shoot 16 days after culture on selective regeneration medium: arrows point to roots. (F) *Cannabis* leaflet coming from a non-transformed hypocotyl-derived shoot after incubation in X-Gluc and decoloration through a graded ethanol series: detail of leaflet outline (right side in panel F). (G) *Cannabis* leaflet from a one-month-old transformed hypocotyl-derived regenerant showing non-uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. (H) Shoot apex from a one-month-old transformed hypocotyl-derived regenerant showing strong and uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series: detail of shoot apical meristem (SAM) after GUS staining (insert in panel H). (I) *Cannabis* leaf from a one-month-old hypocotyl-derived transformed shoot showing uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series: detail of leaflet outline (right side in the panel I). Scale bar (A): 1 mm. Scale bar (B): 0.75 mm. Scale bar (C): 2.16 mm. Scale bar (D): 1.31 mm. Scale bar (E): 4 mm. Scale bar (F): 1 mm; Scale bar of insert (F): 0.5 mm. Scale bar (G): 1.73 mm. Scale bar (H): 2.16 mm; Scale bar of insert (H): 0.5 mm. Scale bar (I): 2.64 mm; Scale bar of insert (I): 0.5 mm.

regenerating shoots (not shown), leaflets coming from the only meristem-derived transformant of the experiment showed a blue coloration distributed along its entire surface (Fig. 6D).

With respect to the response of regenerating shoots after *Agrobacterium* co-culture and culturing in selective regeneration medium containing 100 mg L^{-1} of kanamycin, it should be noted that, regardless of the explant from which they originated, all those transformed regenerating shoots that showed GUS staining signal, developed a normal green phenotype during their development in selective regeneration medium (Fig. 7A). Conversely, some hypocotyls had regenerating shoots with different kanamycin tolerance, as illustrated in Fig. 7B, were kanamycin-resistant (left) and non-resistant (right) regenerating shoots arising from the top of a *Cannabis* hypocotyl can be observed. On the other hand, some kanamycin-non-resistant shoot primordia arising from the basal zone of cotyledons were detected three days after culture on selective regeneration medium (Fig. 7C), although they stopped their development at this stage. In contrast, hypocotyl-derived regenerating shoots showed vigorous growth. Nine-days-old (Fig. 7D), and 20-days-old (Fig. 7E) kanamycin-non-resistant hypocotyl-derived transformants developed on selective regeneration medium and showing an albino phenotype were also observed. Independently of the explant origin of regenerated plants, regenerating shoots were only considered successfully-transformed if they complied with these three conditions: a) green phenotype shown after *in vitro* culture on selective regeneration medium, b) uniform expression (non-chimeric) of the *uidA* gene after incubation of shoot-derived tissue in X-Gluc by GUS histochemical assay, and c) *uidA* and *nptII* gene amplified by PCR (Fig. 8).

4. Discussion

In the present work, we have been able to regenerate *C. sativa* transgenic plants. This represents an important landmark in *C. sativa* breeding and research. Even without addition of plant growth regulators in the culture media employed in the different protocol steps, our results indicate that hypocotyls present higher rates of *in vitro* plant regeneration, transformation, and spontaneous rooting of regenerating shoots compared to the rest of the studied explants. Furthermore, by means of our protocol for *Agrobacterium*-mediated transformation and production of *C. sativa* transgenic plants, hyperhydricity of hypocotyl-derived regenerating shoots was prevented to a great extent. Hypocotyl

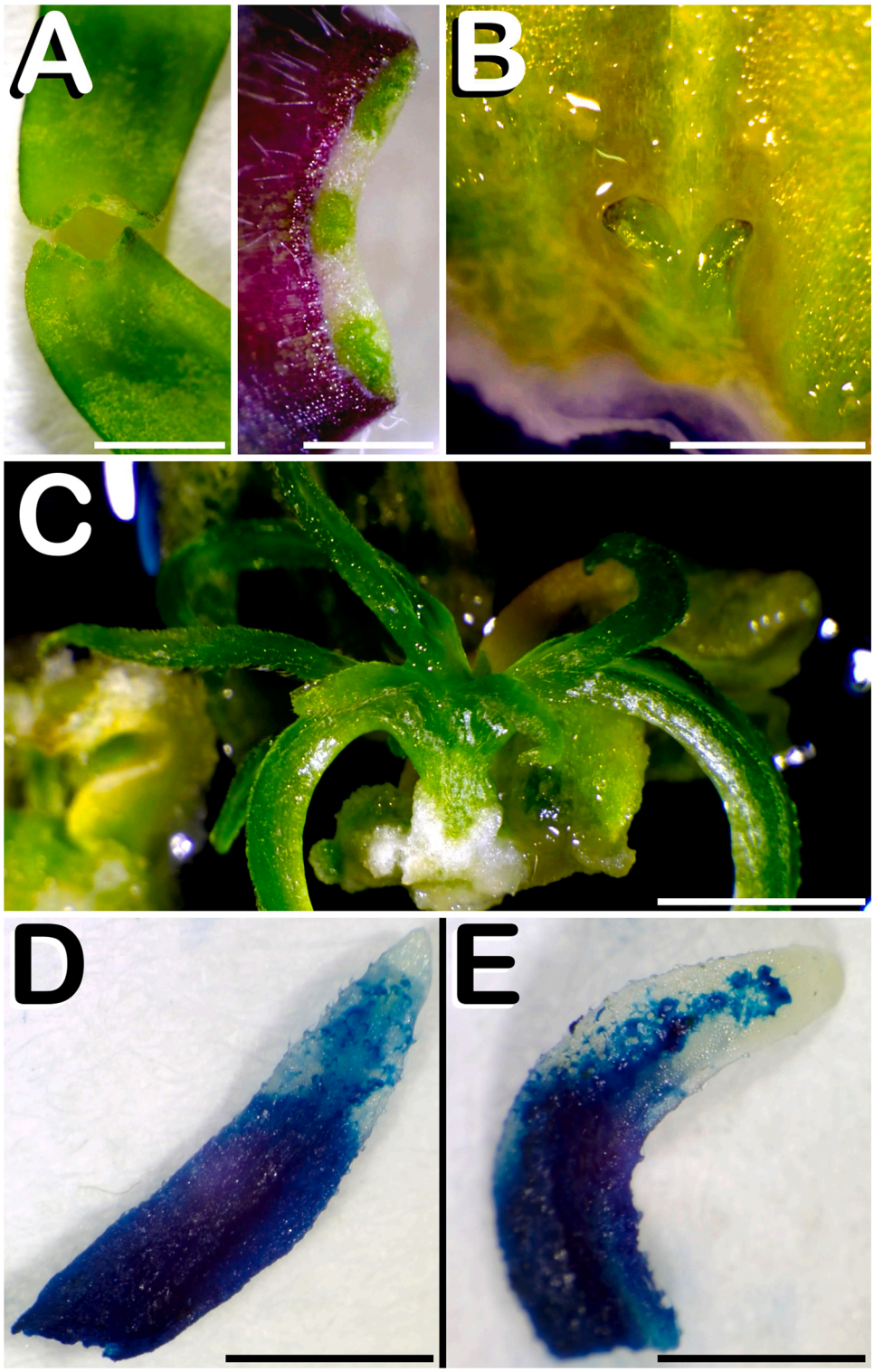


Fig. 5. Genetic transformation of *C. sativa* cotyledon-derived *in vitro* regenerating shoots and GUS staining. The different images are described as follows: (A) Newly dissected cotyledons from a seven-days-old hemp seedling: detail of the transversal section of a newly dissected cotyledon just before co-cultivation with *Agrobacterium* (right side in panel A). (B) Shoot *in vitro* regeneration from *Cannabis* cotyledon after three-day co-cultivation with *Agrobacterium*. (C) *Cannabis* cotyledon-derived transformed shoot 14 days after culture on selective regeneration medium. (D) and (E) *Cannabis* leaflets from a one-month-old transformed cotyledon-derived shoot showing non-uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. Scale bar (A): 2.64 mm; Scale bar of insert (A): 0.65 mm. Scale bar (B): 1 mm. Scale bar (C): 4 mm. Scale bar (D): 1.73 mm. Scale bar (E): 1.73 mm.

explants have been reported as having a superior ability for *in vitro* plant regeneration (Mandolino and Ranalli, 1999; Galán-Ávila et al., 2020; Zhang et al., 2021), and suitability for genetic transformation (Wahby et al., 2013, 2017; Zhang et al., 2021). High plant regeneration ability of *C. sativa* hypocotyls dissected from 7-days-old seedlings, coupled with the putatively unicellular origin of hypocotyl-derived regenerating shoots (Galán-Ávila et al., 2020, and references therein), might have facilitated the genetic transformation of a pluripotent pericycle cell and its subsequent regeneration into a whole transgenic plant, making the difference with previously published attempts concerning transgenic plant regeneration in this species (Feeney and Punja, 2003, 2015; Wahby et al., 2013, 2017). In comparison with the only published report concerning the innovative implementation of the CRISPR/Cas system for genome edition in *C. sativa* species (Zhang et al., 2021), our protocol shows not only an improved efficiency in terms of shoot regeneration and transformation rates, but also a faster capability for *C. sativa* transformed plant production.

The transgenic nature of *C. sativa* regenerated plants was confirmed,

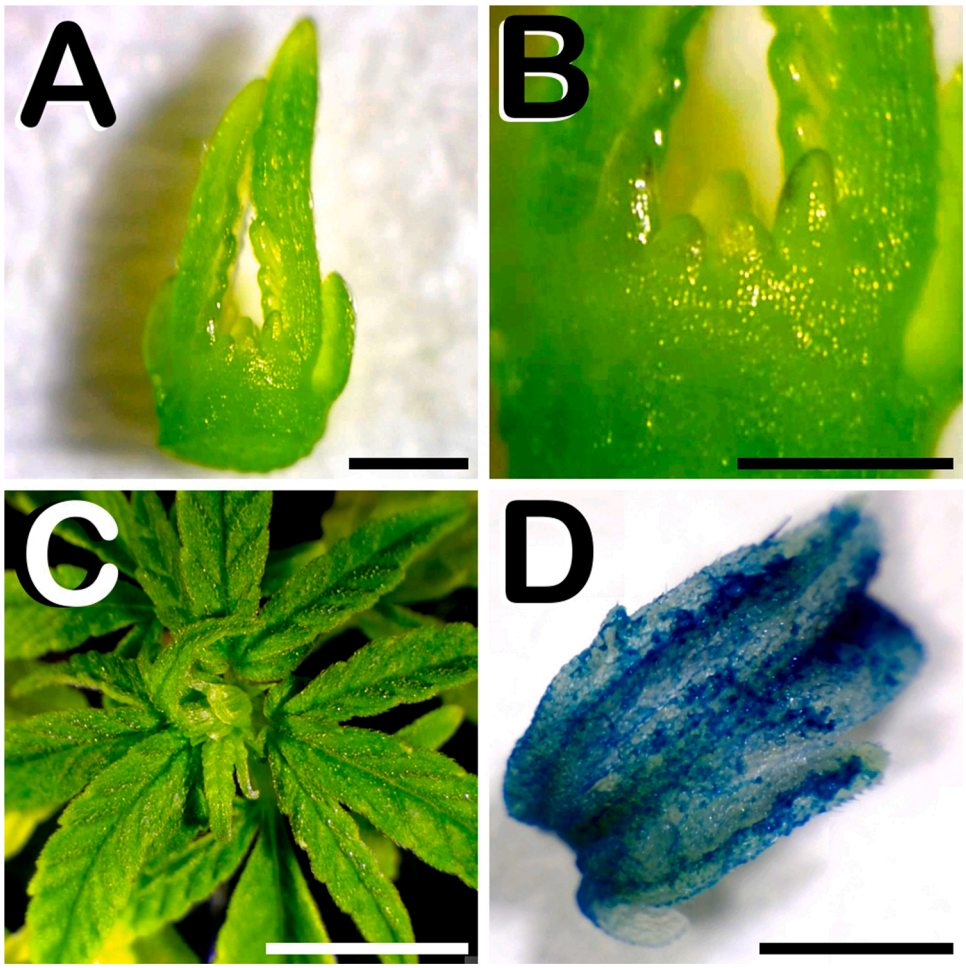


Fig. 6. Genetic transformation of *C. sativa* meristem-derived *in vitro* regenerant and GUS staining. The different images are described as follows: (A) Newly dissected meristem from a seven-days-old hemp seedling. (B) Detail of shoot apical meristem (SAM). (C) Meristem-derived transformed regenerant after 26 days in selective regeneration medium. (D) *Cannabis* leaflet from a one-month-old transformed meristem-derived regenerant showing GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. Scale bar (A): 0.44 mm; Scale bar (B): 0.29 mm; Scale bar (C): 4.00 mm; Scale bar (D): 1.73 mm.

among other techniques, by means of GUS histochemical assay. Employment of *uidA* as a reporter gene in order to validate *Cannabis* transformation constitutes an appropriate strategy, as its expression in different *C. sativa* explants has already been evaluated satisfactorily (Wahby et al., 2013, 2017; Deguchi et al., 2020; Sorokin et al., 2020). In our work, among regenerated plants, putatively chimeric individuals harboring both transformed and non-transformed cells and showing non-uniform GUS staining were also observed. Chimeric transgenic plants have been reported in several species (Ding et al., 2020). Shoot regeneration from a mixture of transformed and untransformed cells, transformed cells conforming only a sector in a shoot, cell cycle arrest of transformed cells, or transient expression of a transgene in some cells of a shoot are possible explanations for the generation of chimeras (Chen, 2011). On the other way, the combined use of *uidA* and *nptII* genes for verifying *Agrobacterium*-mediated plant transformation has been widely implemented in many other species such as *Nicotiana rustica* L. and *Nicotiana tabacum* L. (Hamill et al., 1991), *Solanum lycopersicum* L. (Dan et al., 2006), or more recently, *Gossypium hirsutum* L. (Ahmed et al., 2020b) and *Acmella oleracea* L. (Maggini et al., 2021). Regarding kanamycin resistance *nptII* gene, it should be noted that there are no previous works concerning its expression in *C. sativa*. Here we have demonstrated that, among the rest of the evaluated concentrations, the addition of 100 mg L^{-1} of kanamycin to selective regeneration medium yielded the best results of the dose-response experiment, as it ensured both optimal *in vitro* plant regeneration and spontaneous rooting of regenerating shoots. However, despite the recognized biosafety of *nptII*-derived kanamycin resistance (Nap et al., 1992; Das et al., 2020), and although similar concentrations of kanamycin to those used by us have been employed to successfully select transformants in other species such as *Solanum lycopersicum* L. (Chetty et al., 2013), *Antirrhinum majus* L. (Lian et al., 2020), or *Solanum tuberosum* L. (Bakhsh, 2020), due to the low albinism rate achieved under these experimental conditions, kanamycin-resistance proved to be a poor selectable marker for *C. sativa*. A similar situation has been reported for other species like *Glycine max* L. Merr. (Meurer et al., 1998), or *Phelipanche ramosa* L. (Kullačová and Matúšová, 2020). With respect to the rest of the antibiotics present in selective regeneration medium (250 mg L^{-1} cefotaxime plus 250 mg L^{-1} carbenicillin), their combined use at these concentrations is routinely used for *Agrobacterium* elimination in several *in vitro* plant transformation procedures (da Silva, 2006; Pawar et al., 2013; Yaqoob et al., 2017). Evidence further suggests that antibiotic phytotoxicity can negatively influence explant regenerative ability (Pollock et al., 1983; Qin et al., 2011; Li et al., 2019), as occurs with *Agrobacterium* explant co-culture (Srivastava et al., 2017; de Melo et al., 2020). This might account for the drastic *in vitro* plant regeneration rate reduction registered in our experiments for both *C. sativa* hypocotyl and cotyledon explants.

Cannabis sativa cultivation and research are booming and breeding programs can benefit from the development of transgenic plants, as has occurred with major crops (Herrera-Estrella et al., 2005; Abiri et al., 2015). In addition, new plant breeding techniques such as genome editing offer new avenues for breeding *C. sativa* for medicinal or industrial purposes. Among the most important present challenges is the development of *C. sativa* varieties resistant to biotic (Hadad et al., 2019; Punja et al., 2019; Jerushalmi et al., 2020), and abiotic stresses (Cosentino et al., 2013; Guerriero et al., 2017; Gao et al., 2018; Landi et al., 2019), as well as the development of varieties with specific cannabinoid profiles (Lynch et al., 2016).

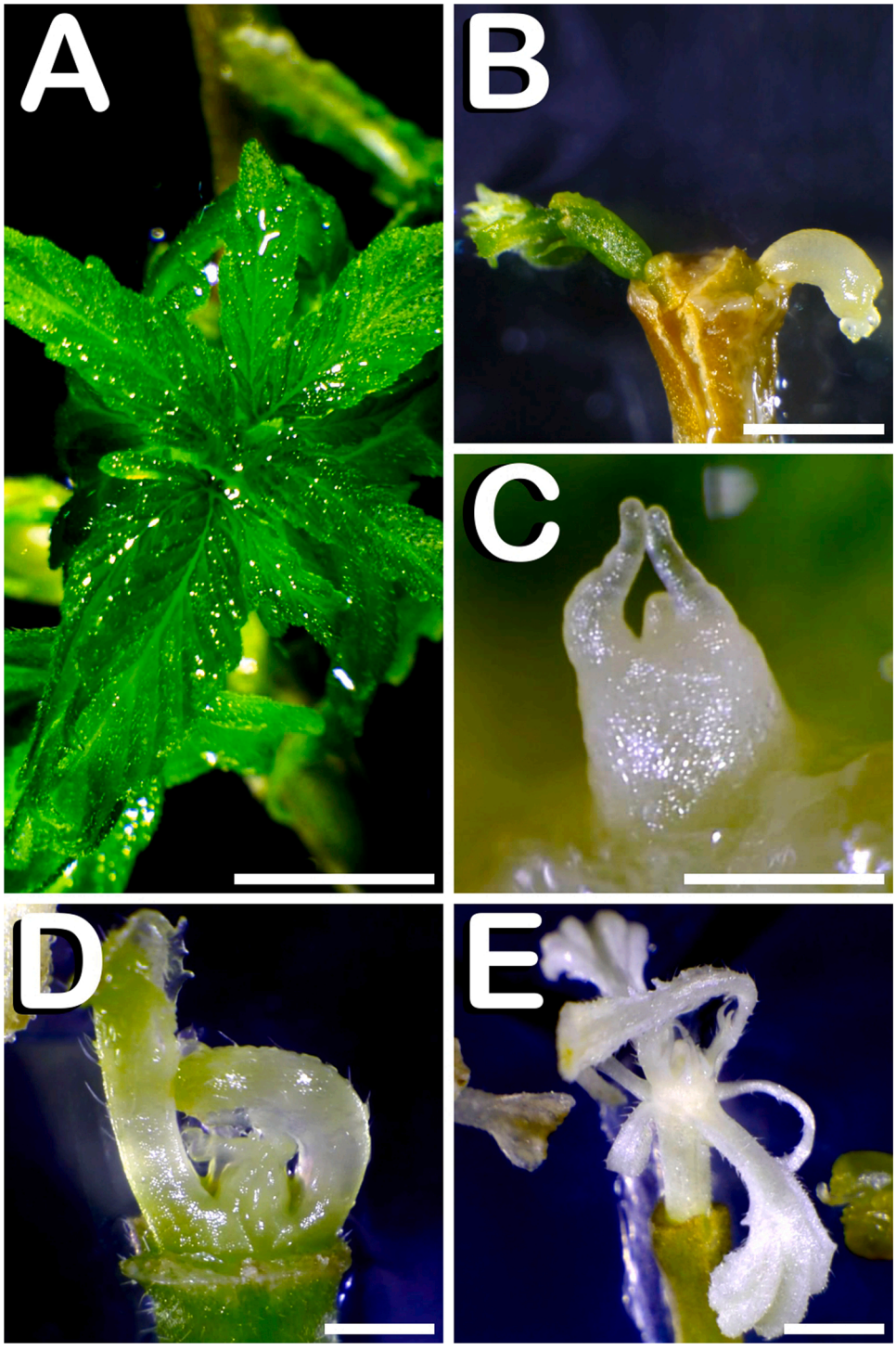


Fig. 7. Kanamycin-resistant and non-resistant phenotypes of *C. sativa* transformants. The different images are described as follows: (A) Kanamycin-resistant hypocotyl-derived regenerant 16 days after culture on selective regeneration medium. (B) Kanamycin-resistant (left) and non-resistant (right) regenerating shoots arising from the top of a *Cannabis* hypocotyl three days after culture on selective regeneration medium. (C) Kanamycin-non-resistant shoot primordium arising from the basal zone of a cotyledon three days after culture on selective regeneration medium. (D) Kanamycin-non-resistant shoot primordium arising from the top of a hypocotyl five days after culture on selective regeneration medium. (E) Kanamycin-non-resistant hypocotyl-derived regenerant 16 days after culture on selective regeneration medium.

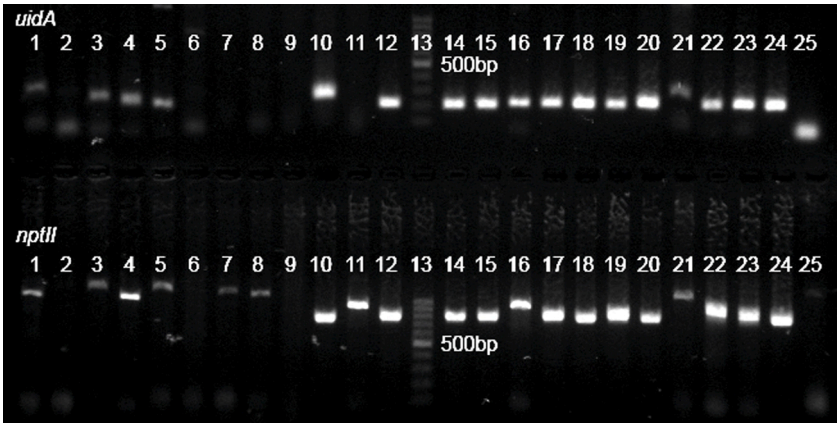


Fig. 8. Polymerase chain reaction (PCR) detection of the *β-glucuronidase* (GUS) (206-bp fragment) and kanamycin resistance neomycin phosphotransferase II (NPTII) (795-bp fragment) genes. Lanes 1-9, 11→ Non-transformed shoots. Lanes 10, 12, 14-20→ Transformed shoots. Lane 13→ 1 kb marker DNA. Lane 21→ DNA from non-transformed control plant. Lanes 22 and 23→ Replicates from lane 10 and lane 18 respectively. Lane 24→ Plasmid. Lane 25→ Negative control.

5. Conclusions

The present work represents a pioneering study documenting the production of *C. sativa* transgenic plants. Our tissue culture-based procedure for *C. sativa* plant genetic transformation could also enable the implementation of genome editing through CRISPR/Cas systems for *C. sativa* breeding, promoting the development of varieties with enhanced agronomic and medicinal properties with industrial and pharmacological utility.

Author contribution statement

A.G.-A., P.G, M.R., J.P. and F.J.H. conceived and designed the research. A.G.-A. and F.J.H. performed the experiments. A.G.-A., P.G, M.R., J.P. and F.J.H. analyzed the results. A.G.-A. wrote the manuscript. A.G.-A., P.G, M.R., J.P. and F.J.H. reviewed and edited the manuscript. All authors have read and approved the manuscript for publication.

Funding

The authors received no specific funding for this work. Pietro Gramazio is grateful to Japan Society for the Promotion of Science (JSPS) for a post-doctoral grant (P19105, FY2019 [Standard]).

Availability of data and material

The datasets used and/or analyzed and plant materials used in the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The author Alberto Galán Ávila declares that his employer (Ploidy and Genomics Ltd.) is seeking a patent over the protocol presented:

- Patent applicant: Ploidy and Genomics Ltd.
- Name of inventor: Alberto Galán Ávila
- PCT Application number: PCT/EP2020/087829 (priority claim dates from 28th of December 2019).
- Status of application: The international search authority of the Patent Cooperation Treaty (PCT) has issued the international search report with its corresponding written opinion certifying the novelty, inventive activity and industrial applicability of the protocol described herein.
- Specific aspect of manuscript covered in patent application: Genetic transformation protocol for the production of *Cannabis sativa* L. transgenic or gene-edited plants.

The rest of authors declare no competing interests.

Acknowledgements

The authors are grateful to Dr Benito Pineda from the Institute for Plant Molecular and Cell Biology (Valencia, Spain) for kindly providing the *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pBIN19.

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