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A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.)

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ABSTRACT

Hemp (*Cannabis sativa*) is an annual multipurpose crop that is distributed worldwide. An efficient regeneration protocol is needed for hemp genetic transformation, micropropagation, and germplasm conservation. We describe here a rapid protocol for in vitro shoot regeneration that uses cotyledons as explants. We concluded that TDZ in MS medium is more efficient in inducing in vitro shoots from cotyledons than BA and ZT. The best result, 51.7% induction frequency and 3.0 shoots per shoot explant, was recorded in MS medium containing 0.4 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA (T4N2). The in vitro shoots grew to the height of 1.5–2 cm in 3–4 weeks after culture initiation. At that time, approximately 80% of the shoots were rooted well in half-strength Ms medium combined with 0.5–2 mg l^{-1} IBA for 4–5 weeks before acclimation. It was observed that younger cotyledons (2–3 days after planting (DAP) were more useful as explants than older ones (5–6 DAP) because they had a significantly higher regeneration frequency. We cultured 3 DAP cotyledons of eight cultivars in T4N2 medium to test the efficiency. The regeneration frequency varied from 35.7% to 54.8%, which indicated that this regeneration protocol was effective, although partly genotype dependent. We regard this protocol as an alternative method for micropropagation and germplasm conservation, and the in vitro plantlets may be suitable to set up a transformation system.

1. Introduction

Hemp (*Cannabis sativa*) is an annual crop with a 6000-year cultivation history. Hemp fibre, seeds and raw materials are used in the textile, oil, paper-making, automotive, construction, bio-fuel, functional food, cosmetics, personal care and pharmaceutical industries (Salentijn et al., 2014). It also could phytoremediate soils polluted by heavy metals (Linger et al., 2005; Shi et al., 2012; Slusarkiewicz-Jarzina et al., 2005). Hemp is suited to various climates around the world, but it was prohibited in the last century in many countries because it is the raw material source of tetrahydrocannabinol (THC), which is a unique secondary metabolite used as a narcotic (De Meijer, 1995). Today, cannabidiol (CBD), which is believed to be an indirect antagonist of THC, is receiving more attention because it is an important pharmacological cannabis with no addictive effect. In recent years, hemp cultivation has been permitted to recover in an increasing number of countries because of its wide use, positive effects on the environment, high caloric value, and high yield, low cultivar input (Papadopoulou et al., 2015). China, Europe and Canada are the most important planting areas for multipurpose hemp. For instance, approximately 800 ha of hemp was planted for fibre and the by-product CBD in the Yunnan province of China in 2013, which greatly benefitted the hemp farmers economically. With this popular trend, both scientists and farmers need new industrial hemp cultivars with multipurpose characteristics (such as a good quantity and quality of fibre or seeds, high CBD content, or monoecious) (Salentijn et al., 2014).

Hemp has a complex genetic background because it is generally dioecious and open pollinated. Current cultivars are bred by traditional methods, including mass selection, crossbreeding, hybrid breeding and marker-assisted breeding. However, genetic

Abbreviations: PGR, plant growth regulator; BA, N6-benzyladenine; NAA, a-naphthaleneacetic acid; TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron); THC, tetrahydrocannabinol; ZT, zeatin; THC, tetrahydrocannabinol; CBD, cannabidiol; IBA, indole-3-butyric acid.

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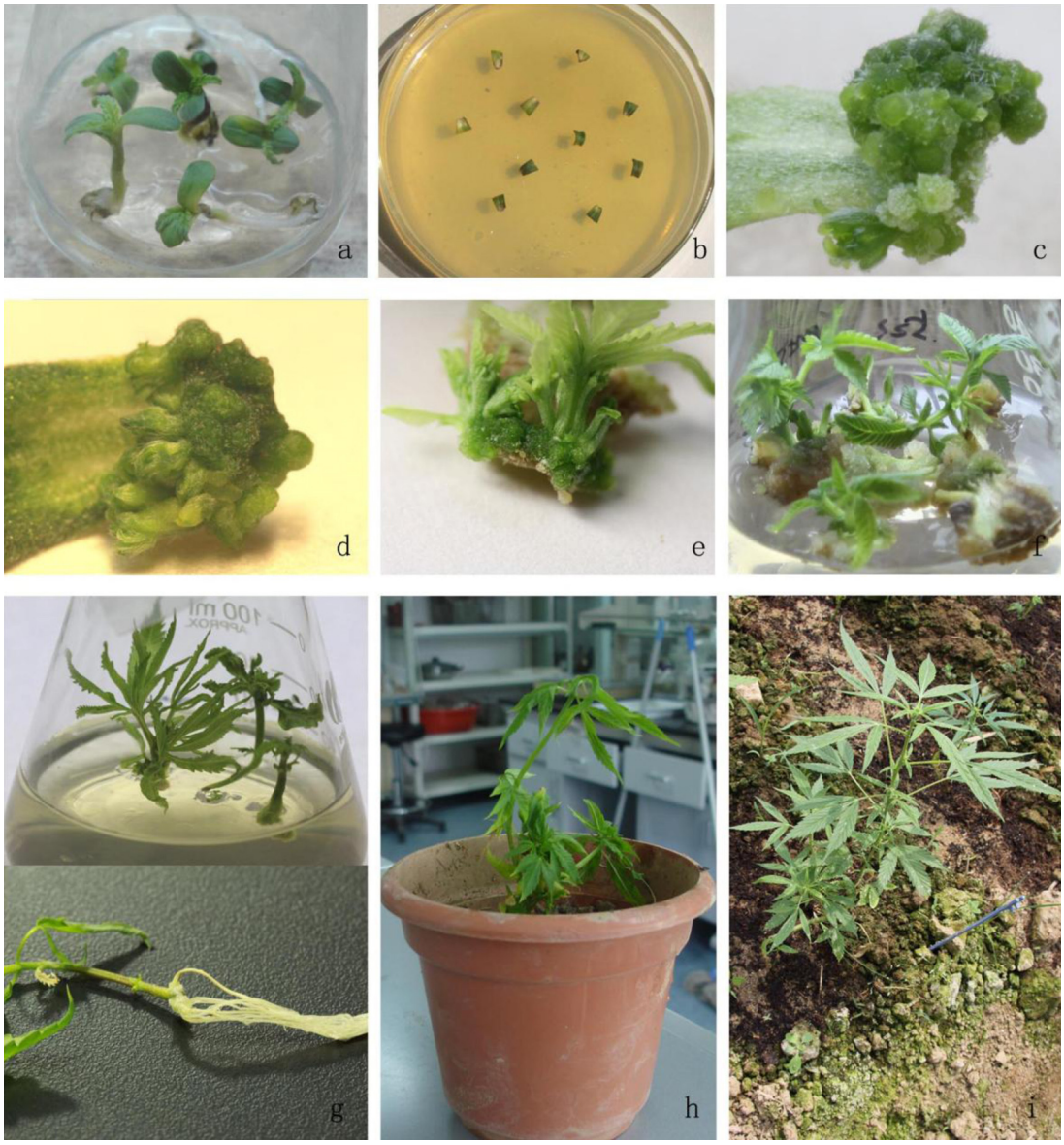

Fig. 1. In vitro plant regeneration of *C. sativa* from cotyledons. (a) 3 DAP sterile seedlings. (b) Cotyledons extracted from seedlings and cut off apex. (c and d) Green global callus arising at peri-axle sites after 5–7 days of culture initiation. (e) Clusters of shoots after 2 weeks of culture initiation. (f) Elongated regenerated shoots. (g) Rooting of elongated shoots cultured on half-strength MS medium containing IBA (0.5–2 mg l^{-1}). (h) Acclimatization of shoots in sterilized organic manure, clay soil and sand (1:1:1). (i) Acclimatization of shoots in garden soil under field conditions.

transformation, a relatively new but efficient breeding method, has not been used successfully in hemp, although it has been used in many other industrial crops (Ye, 2015). An efficient in vitro shoot regeneration protocol is fundamental for genetic transformation. Several regeneration systems have been reported in *C. sativa* recently. For example, apical nodal segments and shoot tips were reported as suitable explants for micropropagation and germplasm conservation, but not as common explants for genetic transformation (Bing et al., 2007; Lata et al., 2009; Wang et al., 2009). Leaves can induce a callus and in vitro shoots in 10 or more weeks (Lata et al., 2010). In the present investigation, we utilized cotyledons as explants to develop a time-saving in vitro shoot regeneration protocol, not only because it is a type of convenient explant source free from cultivation season limitations, but it is also commonly used in genetic transformation research (Kothari et al., 2010,b; Piqueras et al., 2010; Wang et al., 2015).

2. Materials and methods

2.1. Plant material, explant sources and culture conditions

Seeds were harvested from dioecious hemp cultivars in a separate planting garden of the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha. Seeds were soaked in concentrated sulphuric acid for 20 s, washed with tap water for 20 min to soften the episperm, sterilized in 75% (v/v) ethanol for 2 min and 3% (w/v) NaClO for 20 min. After being washed ten times in sterile de-ionized water, the seeds were shelled on a clean bench, and placed onto Murashige & Skoog (MS) medium (Cheng et al., 2011; Grohmann et al., 2011). When the seeds grew up to seedlings, cotyledons were excised as explants to induce in vitro shoots. Seeds and cotyledons were cultivated at 22 ± 2 °C under cool white fluorescent lights (16/8-h photoperiod, 36 μmol m^{-2} s^{-1}).