Industrial Crops & Products 121 (2018) 195–205

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Physio-biochemical characters, embryo regeneration and limonene synthase gene expression in cumin

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ARTICLE INFO

Keywords:
Cumin
Embryo regeneration
Limonene synthase gene expression
Multiple stepwise regression
Physio-biochemical characters

ABSTRACT

Cumin (*Cuminum cyminum* L.) is one of the most important medicinal and spicy herbs in the world. There is no report on the relationships between physio-biochemical characters, embryo regeneration and limonene synthase gene expression in cumin. This experiment was conducted to study physio-biochemical characters, embryo regeneration, limonene synthase gene expression, and their relationship in superior Iranian cumin landraces (Northern Khorasan, Kerman, Semnan, and Golestan) during two years. Results showed that Kerman landrace was superior in terms of embryo regeneration rate (89.33%), limonene synthase gene expression (17.2 fold), proline (3.9 mg/g FW), protein (3.35 mg/g FW), soluble sugars (9.8 mg/g FW), carotenoids (76 mg/g FW), chlorophyll *b* (16.17 mg/g FW), and monoterpene hydrocarbons (34.7%) contents. Embryo regeneration rate was negatively correlated with essential oil ($r = -0.78^{*}$) and it was positively correlated with limonene synthase gene expression ($r = 0.94^{**}$) and physio-biochemical characters including peroxidase activity ($r = 0.95^{**}$) and the contents of proline ($r = 0.82^{*}$), protein ($r = 0.88^{*}$), soluble sugars ($r = 0.99^{**}$), carotenoids ($r = 0.97^{**}$), chlorophyll *a* ($r = 0.95^{**}$), chlorophyll *b* ($r = 0.84^{*}$), chlorophyll *a* + *b* ($r = 0.91^{**}$), limonene ($r = 0.94^{**}$), and monoterpene hydrocarbons ($r = 0.93^{**}$). There was a positive and significant correlation between embryo regeneration rate and altitude ($r = 0.75^{*}$). So it is concluded that embryo regeneration of cumin landraces are dependent of changes in altitude of their origins. The positive regression and correlation coefficients of soluble sugars with embryo regeneration rate indicate that this character is a suitable selective index for improving embryo regeneration rate. Considering the narrow-sense heritability of soluble sugars, this character can be used in breeding of embryo regeneration.

1. Introduction

Cumin (*Cuminum cyminum* L.) is one of the most important medicinal herbs in the world which belongs to the Apiaceae. The Apiaceae family consists 455 genera and over 3500 species and it is regarded as one of the largest plant families. Cumin was reported as a plant having pharmaceutical and medical importance by historical evidence and recent studies (Baranski, 2008; Dubey et al., 2017). It was reported that cumin oil increases the glutathione production in human tissue up to 700%. Glutathione is the first defense line of the body against oxidative damage and it is a key compound for assisting the body, particularly the liver and kidneys in the removal of noxious chemicals. Cumin oil has medical importance such as enhancing appetite, taste perception, digestion, and therapy of fever, diarrhea, vomiting, abdominal distension, edema, and puerperal disorders. (Pandey et al., 2015; Dubey et al., 2017).

An effective and reproducible *in vitro* regeneration system is required for studies on genetic manipulation in each plant species (Ebrahimie et al., 2006). Studies have shown that various genotypes of a plant species have different regeneration capacity depending on genetic and biochemical properties, ability of key elements in regeneration path and also endogenous phytohormones metabolism (Ebrahimie et al., 2006; Dodig et al., 2008; Jariteh et al., 2015). Studies indicated that plant regeneration through callus production is time-consuming

Abbreviations: MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; BAP, 6-benzylaminopurine; ANOVA, analysis of variance; SE, standard error; qPCR, quantitative polymerase chain reaction; bp, base pair; Chl, chlorophyll; UV, ultra violet; cDNA, complementary DNA

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https://doi.org/10.1016/j.indcrop.2018.05.006
Received 21 October 2017; Received in revised form 8 April 2018; Accepted 4 May 2018

and it depends on landraces and also it leads to abnormal genetic changes and somaclonal variation in regenerated plants (Ebrahimie et al., 2006; Pandey et al., 2016). Embryos as explants are more economically advantageous than other explants since they are free of time-consuming processes of callus formation, successive sub-culture, and somaclonal variation (Ebrahimie et al., 2006). Unfortunately, the tissue culture response of genotypes cannot be determined before experiment which is very costly, laborious, and time-consuming (Dodig et al., 2008). Therefore, some researchers predict the results of tissue culture using correlations between characters and tissue culture properties in advance to avoid experiment with a large number of genotypes in tissue culture stage. The researchers suggested that it is possible to screen the genotypes with desirable tissue culture properties even at the agronomic stage which is relatively simple (Li et al., 2003; Haliloglu et al., 2005; Dodig et al., 2008). In various studies, biochemical characters including the content of proline, protein, soluble sugars, and antioxidant enzymes have been used as markers to evaluate tissue culture and regeneration (Jeyaseelan and Rao, 2005; Jariteh et al., 2015; Abbasi et al., 2016). Studies show that antioxidant enzymes such as peroxidase play important roles in plants growth and differentiation and their high activity affects cellular processes such as regeneration (Thakar and Bhargava, 1999; Molassiotis et al., 2004; Jariteh et al., 2015). Essential oils and their compositions particularly alcohols and phenols are genetic properties negatively affecting regeneration process (Reis et al., 2008; Ptak et al., 2013). Many researchers have attempted to analyze essential oil composition of cumin seeds. They found major components including terpenic hydrocarbons, cumin aldehyde, polyphenol, and flavonoids as an important biologically active dietary, and medicinal compounds (Hajlaoui et al., 2010; Dubey et al., 2017). Also, limonene, alpha, beta-Pinene, and some other terpenic compositions were found in cumin oil (Johri, 2011). Terpenic hydrocarbons of plants essential oil are genetic property affecting on plant regeneration (Reis et al., 2008, Alonzo et al., 2001). Ebrahimie et al. (2006) produced a lot of shoot without callus formation and subculture by regeneration process using cumin embryo as an explant. Pandey et al. (2013) suggested the method of direct regeneration without callus formation using cumin embryos and they used this method for β-glucuronidase transgenic cumin. Although, few studies have been conducted on embryo culture and its regeneration in cumin, there is no report on relationships between physio-biochemical characters, limonene synthase gene expression and cumin regeneration. Therefore, the current study was aimed to evaluate physio-biochemical characters, embryo regeneration and limonene synthase gene expression in superior Iranian cumin landraces and then study the relationships between physio-biochemical characters and embryo regeneration using correlation coefficient and multiple regression. These characters can be employed as markers to predict embryo regeneration in cumin.

2. Materials and methods

2.1. Plant samples

Four superior Iranian cumin landraces including Northern Khorasan, Kerman, Semnan, and Golestan (Fig. 1, Table 1) were selected based on having high essential oil, 1000-seed weight, seed weight per plot, and seed per umbellet during two growing seasons of 2014–15 (Bahmankar et al., 2015). Seeds of cumin landraces were provided by seed bank of Aburaihan College, Tehran University. This seed bank was established by the second author of the current study in 2011 by collecting landraces of main producer provinces from Iran including Kerman, Semnan, Yazd, Isfahan, Golestan, North Khorasan, South Khorasan, and Razavi Khorasan provinces. Cumin plants were propagated and seeds were collected and then experiments were done. The present experiments were divided into three sets including measurement of physio-biochemical characters, embryo regeneration, and limonene synthase gene expression.

2.2. Experiment of physio-biochemical characters

In the first experiment, leaf tissue samples of four superior cumin landraces were immediately frozen in liquid nitrogen to measure physio-biochemical characteristics. Each replication was the average of three sampled plants and 10 leaves were sampled from each plant. Chemicals required for culture media, plant hormones, and all standards and reagents were purchased from Merck Co. (Germany) and Sigma-Aldrich (USA).

2.2.1. Measurement of protein

About 1 g of leaves were homogenized in 1 M Tris-HCl (pH 6.8) and 2.5% (w/v) polyvinylpolypyrrolidone (PVPP) (to avoid phenol oxidative effects) at 4 °C by a mortar. The homogenates were centrifuged at 4000 rpm for 30 min at 4 °C (2 times) and supernatants were kept at −70 °C till protein determination. The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.2.2. Measurement of proline

First 0.1 g of leaf tissue was homogenized in 10 ml of aqueous sulphosalicylic acid and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant (2 ml) was mixed with 2 ml of each acid ninhydrin and glacial acetic acid followed by incubation at 70 °C for 1 h. The reaction mixture was cooled on ice cold water and 4 ml of toluene was added. The upper phase was separated and absorbance was determined at 520 nm against a blank. Finally, the proline concentration was determined from a standard curve (a calibration curve was obtained with L-proline as the standard, Bates et al., 1973).

2.2.3. Measurement of soluble sugars

About 0.1 g of plant tissue sample was homogenized in 10 ml ethanol (80%, v/v) and centrifuged at 4000 rpm for 20 min (two times). The supernatant was collected and known amount of ethanol extract (0.1–0.2 ml) was evaporated to dryness in a test tube on a water bath and cool it to room temperature. One ml of distilled water was added to each test tube and mixed thoroughly. To each test tube, 4 ml of anthrone reagent (0.2%, w/v anthrone in concentrated H_2SO_4) was added along the wall of the test tube and mixed gently, heated on a water bath at 100 °C for 10 min, cooled rapidly under running cold water and absorbance was measured at 620 nm against reagent blank. The amount of total soluble sugars was calculated using standard curve prepared from the graded concentration of glucose (Yemm and Willis, 1954).

2.2.4. Measurement of peroxidase activity

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Hemeda and Klein (1990). The mixture consisted of 1 ml of 50 mM potassium phosphate buffer (pH 6.6), 0.1 ml H_2O_2 (3%), 90 µl guaiacol (1%), and 10 µl enzyme extract. The increase of absorbance was recorded at 470 nm. The POX activity was reported as µM of H_2O_2 decomposed per min per mg protein (Unit mg protein).

2.2.5. Measurement of photosynthetic pigments contents

About 0.1 g of fresh leaves was ground in 80% acetone by a mortar. The homogenate was centrifuged at 4000 rpm for 10 min at 4 °C to remove cell debris. Supernatants were collected and absorbance was recorded at 470, 646.8, and 664 nm using a UV–vis spectrophotometer. The contents of photosynthetic pigments (PSP) (Chl *a*, Chl *b*, and carotenoids (xanthophylls and carotenes (x + c)) were calculated according to Lichtenthaler and Buschman (2001) equations.

2.2.6. Essential oil extraction

The dried powdered cumin seeds (40 g) were placed in a Clevenger apparatus with 400 ml of hydro-distilled water for three hours (Hajlaoui et al., 2010). Then, the essential oil content was determined based on dry weight and expressed as percent. Essential oil