Bangladesh J. Bot. **48**(3): 575-581, 2019 (September)

IN VITRO PROPAGATION OF THE CHINESE TRADITIONAL AND MEDICINAL PLANT *HERACLEUM SCABRIDUM* FRANCH

LI-JUAN ZOU, JIN-YAO HU, MING-HUA LUO AND QING-GUI WU*

Ecological Security and Protection Key Laboratory of Sichuan Province, Mianyang Normal University, Mianyang, 621000, China

Keywords: *Heracleum scabridum*, Multiple shoot, Organogenesis

Abstract

Three explants such as stem tips, leaves and petioles of *Heracleum scabridum* were compared for their shoot development/differentiation ability by using different plant growth regulators (PGRs). The most effective PGRs combination for callus induction and organogenesis was determined. TDZ, Kn, Zn and IAA were used to induce multiple shoots. For indirect organogenesis (from the calli), the best response (27.25 ± 4.19 shoot per stem tips) and (18.23 ± 2.12 per leaves) were obtained in Murashige and Skoog (MS) medium fortified with 0.5 mg/l IAA and 3.0 mg/l Zn with 100, 97.3% induction rate, respectively. MS medium containing 0.5 mg/l IAA and 3.0 mg/l Zn was also found to be optimal for shoot regeneration from petioles. The highest percentage of regeneration (94.6) with mean number of shoots 17.83 ± 4.87 from petioles were produced. For direct organogenesis (from axillary bud shoot clumps), 0.1 mg/l IAA and 1.5 mg/l TDZ were found to be optimal for shoot regeneration of stem tips, with mean numbers of axillary bud shoot clumps 7.12 ± 1.23 were produced. Plantlets were transferred to soil with 95% of plants acclimatized recovered successfully.

Introduction

Heracleum scabridum Franch, an herbaceous perennial plant, belonging to the Umbelliferae is unique to China. *H. scabridum*, also locally known as "Dianbaizhi", is one of the most important of the traditional Chinese medicines and is mainly distributed in Yunnan province of China (Shen *et al.* 2015), specially in northern mountainous regions with an altitude ranging from 1500 - 2500 m. It has been widely used in traditional medicine systems as it possesses many antioxidant and antibacterial properties due to its abundant secondary metabolites, some coumarin derivatives and furanocoumarins such as isobergapten (XVII), pimpinellin (XVIII), sphondin (XIX), imperatio, deltoin and so on (Sun *et al.* 1978). Recent study showed that 16 compounds were isolated from *Heracleum scabridum* and identified, especially, some compounds are isolated from this plant for the first time (Wei *et al.* 2017). As a traditional Chinese medicine, it can dispel wind, expel cold, relieve pain, carminative, remove dampness, gynecological diseases, it has been proved that the petroleum ether extract had antipyretic, analgesic activities by pharmacological experiments (Niu *et al.* 2002). *Heracleum* species is characterized as an essential oil rich plant and medicinal plant as well. Due to *Heracleum* potent antioxidant properties, the present study has been focused on their medicinal components, especially their phytochemical constituents (Liu *et al.* 1998, Tkachenko 2010), antimicrobial activities (Souri *et al.* 2004, Torbati *et al.* 2014). Various studies have reported essential oil (EO) composition according to biological activities, such as octylacetate and hexyl butyrate, hexyl-2-methylbutanoate and hexyl isobutyrate. (E)-anethole β-pinene was reported in leaves EO or roots (Torbati *et al.* 2014). However, until now, a few reports have focused on other physiological characteristics of *H. scabridum*, there has many areas of research unknown and this plant has great potential for research in future because of its various properties. As its medicinal value is constantly being excavated, it will attract attention of increasing number of workers. As an

*Author for correspondence: <qgwu30@163.com>.

important source of high medicinal value and unique geographical distribution, it has been subjected to heavy collection from the wilds. Thus, to fulfill the demand of the pharmaceutical industry and other related studies, the main goal of this work was to establish a standard protocol for regeneration in *H. scabridum*.

Plant tissue culture techniques have been used as an efficient approach for the mass propagation and conservation of some rare and endangered species, especially those with high medicinal or economic value. To date, work on *in vitro* clonal propagation of *H. scabridum* has not been reported. The effect of three different cytokinins with different concentrations and compositions was studied on the callusogenesis of explants, and the efficiency of explants on shoot formation. Shoot induction and mean shoot number from various explants exhibited different responses. The shoots obtained were rooted, and the plantlets obtained were acclimatized.

Materials and Methods

Mature fruits of *Heracleum. scabridum* were obtained from Dingqing County, Changdu, Tibet, China. The species was identified by Professor Ming-hua Luo, Mianyang Normal University. Seeds were soaked in warm water for 8 - 12 hrs before sowing. Stem tips, leaves and petioles were used as explants. The explants were washed in running tap water for about 15 min to remove microbes. Thereafter, in aseptic conditions, the explants were surface-sterilized in 75% (v/v) ethanol for 30 sec, soaked in 1.5% (v/v) sodium hypochlorite solution for about 10 - 15 min, and rinsed 3 - 5 times with sterilized distilled water. Surface sterilized explants were aseptically inoculated in MS medium with 2% (w/v) sucrose and 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8. The medium was then autoclaved at 121°C for 20 min. All cultures were maintained at 24 ± 1°C under a 16/8 hrs light/dark cycles of medium light (100 μmol m^{-2} s^{-1}). MS basal medium was used for all stages of micropropagation.

Assays were variously supplemented with different PGRs (IAA, IBA, TDZ, Zn and Kn). Thirty explants were tested for each treatment and each experiment was replicated three times. Shoot regeneration and the number of shoots per explant were recorded after 4 weeks.

Adventitious shoot clumps were transferred equally to different half strength MS media combinations containing IBA or no PGRs to induce roots. Thereafter, rooted plantlets were removed from *in vitro* culture, washed thoroughly with distilled water to eliminate debris and planted in plastic pots containing peat and vermiculite (3 : 1 v/v), and covered with transparent plastic to maintain 80% humidity.

The data were statistically analyzed using one-way ANOVA. DMRT was applied at the 0.05 level of probability to compare individuals within a treatment (Gomez and Gomez 1984) using SPSS18.0 software.

Results and Discussion

Stem tips, leaves and petioles (±1 cm) were isolated and cultured in MS media containing various concentrations of PGRs. Three types of explants cultured on different media showed different responses (Table 1). The frequency of shoot induction and mean number of shoots are presented in Table 1.

Initially, stem tip explants became swollen, after 7 days, loose and pea-green callus appeared on the base of stem tips, and continued to develop for 7 days, regenerated adventitious buds arose from the callus (Fig. 1 A1, A2). As the culture time was extended, one clump of adventitious buds eventually developed from one callus clump (Fig. 1A3, A4). The data revealed the significant differences in shoot induction and mean shoot numbers among the different treatments. Calli and

shoots were all induced by stem tips explants on medium containing TDZ, zeatin or Kn alone. No response was observed in control media. The combination of IAA and zeatin promoted a significant increase in callus and shoot formation relative to that induced by the combinations of IAA and Kn. MS supplemented with 0.5 mg/l IAA and 3.0 mg/l to Zn was the most effective combination for stem tips in all treatments, with 27.25 ± 4.19 shoots were produced per explants on average.

Table 1. Effect of PGRs on shoot regeneration of *H. scabridum* after 4 weeks of culture.

PGRs (mg/l)				Callus induction (%)			Mean shoot number		
IAA	TDZ	Kn	Zn	Stem tip	Leaf	Petiole	Stem tip	Leaf	petiole
0	0	0	0	0g	0e	0f	0h	0e	0g
0	1.0	0	0	63.8e	0e	0f	5.26 ± 0.58f	0e	0g
0.5	1.0	0	0	79.3c	65.2d	63.9e	8.71 ± 1.35d	4.71 ± 1.54d	3.56 ± 0.78f
0.5	1.5	0	0	84.2b	77.9c	73.1d	9.27 ± 2.07cd	5.12 ± 0.98d	4.67 ± 1.12f
0.5	2.0	0	0	80.5c	68.6d	66.8e	8.52 ± 2.21d	4.69 ± 0.72d	4.35 ± 0.98e
0	0	1.0	0	48.1f	0e	0f	3.23 ± 0.68g	0e	0g
0.5	0	1.0	0	82.4c	71.2d	67.9e	11.21 ± 2.35d	5.72 ± 1.23d	5.18 ± 1.43ef
0.5	0	2.0	0	90.5b	83.1c	89.2b	13.23 ± 1.34cd	6.43 ± 0.66d	6.34 ± 2.83de
0.5	0	3.0	0	88.7b	79.4c	78.3d	12.26 ± 2.58cd	5.88 ± 1.19d	5.91 ± 2.36ef
0	0	0	1.0	70.1d	0e	0f	8.12 ± 1.32e	0e	0g
0.5	0	0	1.0	85.8b	82.7c	80.1c	15.34 ± 2.56c	9.47 ± 2.13c	8.36 ± 2.13d
0.5	0	0	2.0	91.4b	91.2ab	88.1b	21.2 ± 4.28b	13.17 ± 1.21b	14.37 ± 4.31b
0.5	0	0	3.0	100a	97.3a	94.6a	27.25 ± 4.19a	18.23 ± 2.12a	17.83 ± 4.87a
0.5	0	0	4.0	79.1c	85.4c	82.4c	15.34 ± 3.27c	10.25 ± 2.71b	11.56 ± 2.34c
0.1	0	0	1.0	77.5c	62.5d	62.1e	9.72 ± 1.38cd	4.45 ± 0.76d	5.78 ± 0.74ef
0.1	0	0	2.0	83.1c	67.8d	77.4d	13.77 ± 1.76cd	6.78 ± 0.49d	7.53 ± 0.63de
0.1	0	0	3.0	85.2b	72.9d	79.4d	11.22 ± 2.12cd	5.79 ± 1.12d	6.87 ± 1.17de

Means followed by different letters in a column are statistically significant at $p < 0.05$.

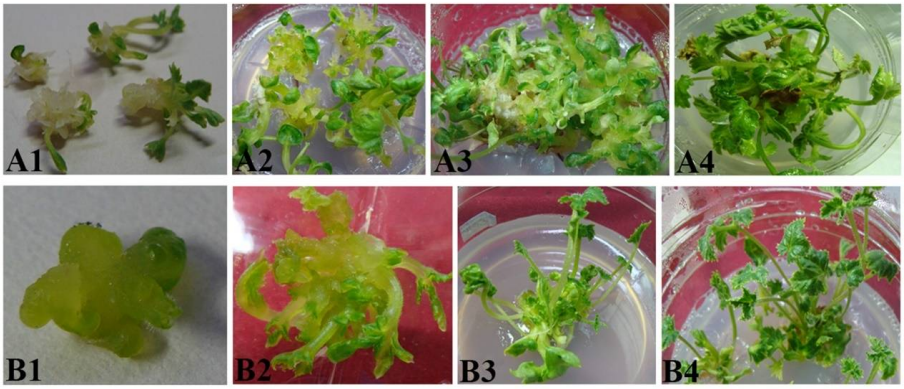

Fig. 1A1-A4. Callus development and organogenesis from stem tips explants of *H. scabridum* cultured on MS with 0.5 mg/l IAA and 3.0 mg/l Zn, B1. Callus formation from leaves, B2-B4. Organogenesis from leaves explants cultured on MS with 0.5 mg/l IAA and 3.0 mg/l Zn.

The leaf explants initially enlarged on the inductive medium within the first 7 days of culture. They subsequently became swollen and curled, yellowish green callus appeared from the cut zones of leaves explants. Thereafter, regenerated shoots arose from the callus from the cut margins after 3 weeks in culture, and its development was recorded (Fig. 1 B1, B4). As shown in Table 1, calli developed on medium containing TDZ, Kn or Zn alone did not show any sign of regeneration and as such IAA was required for calli and shoot induction. Significant differences in the rate of callus formation and organogenesis were observed in leaf explants, with the highest rate in 0.5 mg/l IAA and 3.0 mg/l Zn, which yielded 18.23±2.12 shoots per explant (Table 1).

Petiole explants also showed a good response for callus formation. After 7 days, on both ends of the explants were covered with loose and pea-green callus. Thereafter, shoots arose from the callus after 2 weeks (Fig. 2A). Differences in the rate of callus formation and organogenesis were observed in petiole explants. Highest rate was obtained with 0.5 mg/l IAA and 3.0 mg/l Zn, which yielded 17.83 ± 4.87 shoots per explant.

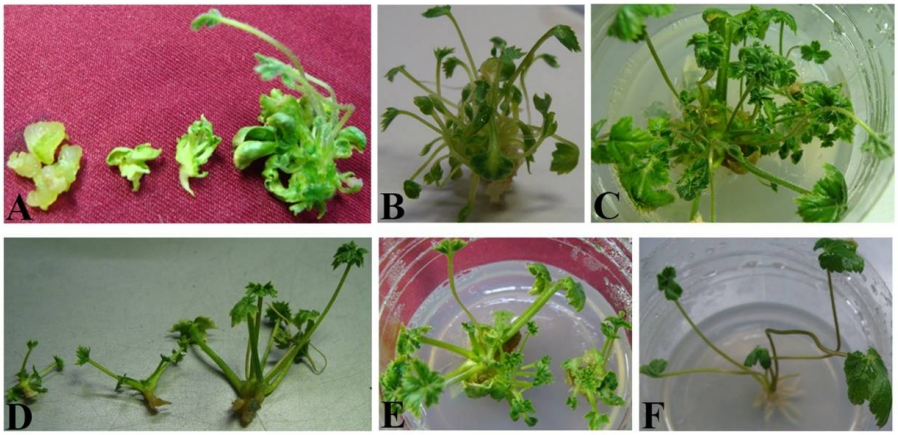

Fig. 2A. Callus development and organogenesis from petioles of *H. scabridum* on MS with 0.5 mg/l IAA and 3.0 mg/l Zn. B and C, shoot multiplication of *H. scabridum*, D and E represent direct organogenesis by stem tips explants and F. rooting.

Shoots developed from stem tip explants of *H. scabridum* in two different ways: Indirect organogenesis (from the calli, Fig.1 A1-A4) and direct organogenesis (from axillary bud shoot clumps, Fig. 2D). Axillary bud shoot clumps were all induced by explants on medium containing TDZ or zeatin alone. Direct organogenesis was also the main mode of induction observed, with an optimum combination of 0.1 mg/l IAA plus1.5 mg/l TDZ, which produced an average number of shoots per explants of 7.12 ± 1.23, with a 94.4% induction rate (Table 2). A maximum number of shoots was achieved in MS medium with 1.5 mg/l TDZ, however, addition of more than 1.5 mg/l TDZ decreased the mean number of shoots per explant. These data suggested that TDZ had a greater effect on shoot regeneration in *H. scabridum* than Zn. MS medium with 0.5 mg/l IAA plus 1.0 mg/l TDZ was optimal for axillary bud shoot clumps proliferation. A large scale regenerated shoots within 20 days were obtained (Fig. 2E).

The excised axillary shoots larger than 4.0 cm in length were transferred to rooting media for root induction. After 30 days of incubation, the percentage of root induction and the number of root per shoot were recorded. Present results showed that IBA was suitable for root regeneration of

H. scabridum and no roots developed in auxin (control). In contrast, all the media containing IBA induced rooting (Table 3).The optimal medium for rooting contained 0.8 mg/l IBA, in which 95.7% of root induction (Fig. 2F). The mean number of roots per shoot was 20.73, with an average root length of 6.1 mm. A further increase in IBA concentration above 1.0 mg/l resulted in slow root development, and the number of roots per plant decreased to 4.83 (Table 3). After 4 weeks of culture, well-developed plantlets were transferred to plastic pots containing peat and vermiculite (3 : 1 v/v), with a 95% survival rate (data not shown).

Table 2. Effect of PGRs on shoot regeneration from stem tips of *H. scabridum* after 4 weeks of culture.

PGRs (mg/l)			Stem tips	
IAA	TDZ	Zn	Shoot induction (%)	No. of shoots
0	0	0	0f	0d
0	1.0	0	72.4d	3.38 ± 0.39c
0	1.5	0	88.3b	5.02 ± 2.04b
0	2.0	0	80.6c	3.18 ± 0.37c
0	0	1.0	63.1e	2.15 ± 0.37c
0	0	2.0	71.9d	3.27 ± 0.37c
0.1	0.5	0	81.8c	4.26 ± 0.17b
0.1	1.0	0	82.9c	4.48 ± 0.32b
0.1	1.5	0	94.4%a	7.12 ± 1.23a
0.1	2.0	0	87.2b	5.34 ± 0.48b
0.5	0	1.0	63.9e	2.18 ± 0.23c
0.5	0	1.5	73.6d	3.15 ± 0.37c

Means followed by different letters in a column are statistically significant at $p < 0.05$.

Table 3. Root formation in *H. scabridum* on different rooting medium.

Treatment (mg/l)	Rooting (%)	No. roots/regenerated shoot	Root length (mm)
0	0e	0e	0e
IBA 0.2	84.2b	11.67 ± 1.92b	4.2 ± 0.71b
IBA 0.8	95.7a	20.73 ± 4.86a	6.1 ± 1.26a
IBA 1.0	42.5c	4.83 ± 0.52c	2.2 ± 0.54c
IBA 1.2	25.8d	4.16 ± 0.68c	1.8 ± 0.47c

Means followed by different letters in a column are statistically significant at $p < 0.05$.

To date, the reports on the tissue culture of *H. scabridum* are not available. This is the first time that authors reported organogenesis from three types explants and in two different ways. In the present assay, shoot regeneration was successfully induced from three types of explants of *H. scabridum* at a high frequency. Significant differences existed in mean shoot numbers among the three explants types (stem tips > leaves > petiole). Micropropagation via direct or indirect organogenesis from different explants has been used for regeneration of many species, with different plant growth regulators (Vasil and Thorpe 1994). In this study, organogenesis was successfully generated from stem tips explants of *H. scabridum* in two different ways (namely, direct organogenesis, indirect organogenesis) with different PGRs combination. Zn, TDZ and Kn

are commonly used for shoot regeneration in many plant species (Ellis *et al*. 1991 and Huetteman and Preece 1993, Kou *et al*. 2012). It is found that *H. scabridum* stem tips explants were more sensitive to Zn than TDZ or Kn (Table 1), all the cytokinins Zn, TDZ or Kn) alone did not induce calli from leaves and petioles explants. When these were combined with IAA in callus- induction media, they increased the callus induction percentage and mean shoots numbers, indicating that IAA was a key factor in inducing callus formation and organogenesis. Zn, TDZ or Kn were effective at inducing callus formation in *H. scabridum* stem tips, leaves and petioles, but the combination of Zn with IAA generated higher frequencies of callus and increased mean shoots compared TDZ or Kn.

Some recent reports have shown that TDZ has both cytokinin and auxin functions and thus had been used increasingly in plant regeneration (Guo *et al*. 2011 and Guo *et al*. 2012). The present experiments showed similar results,in the direct organogenesis from stem tips explants of *H. scabridum*, with TDZ alone, the shoot induction can reach 88.3% and TDZ had a greater effect than that Zn for the activation of axillary buds (Table 2). TDZ was reported to induce callus formation in a variety of plant culture systems with a higher rate than other growth regulators (Capelle *et al*. 1983, Murthy *et al*. 1998), these findings are also in agreement with the present study. Generally, a high cytokinin-to-auxin ratio results in shoot induction (Krikorian 1995), but a higher ratio causes abnormal growth (Singh *et al*. 2013), which is in conformity with the present study, when TDZ concentration was increased to 1.5 mg/l gave a better response, but a higher concentration (2.0 mg/l) was toxic to the explants and caused hyperhydricity. The present results indicated that a lower concentration of TDZ (1.5 mg/l) in combination with 0.1 mg/l of IAA lead to a higher frequency of direct organogenesis of stem tips explants of *H. scabridum* (Table 2).

Acknowledgments

The work was supported by Applied Basic Project of Sichuan Science and Technology Department (2018JY0192, 2018JY0244), Scientific research initiation project of Mianyang normal university (QD2019A12), the National Natural Science Foundation of China (31100246) and the Open Project from the Ecological Security and Protection Key Laboratory of Sichuan Province (ESP1602).

References

Capelle SC, Mok DW, Kirchner SC and Mok MC 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N-(delta-isopentenyl)[8-C] adenosine in callus tissues of *Phaseolus lunatus* L. Plant Physiol. **73**: 796-802.

Ellis DD, Barczynska H, McCown BH and Nelson N 1991. A comparison of BA, zeatin and thidiazuron for adventitious bud formation from Piceaglauca embryos and epicotyl explants. Plant Cell Tiss. Org. Cult. **27**: 281-287.

Guo B, Abbasi BH, Zeb A, Xu LL and Wei YH 2011. Thidiazuron: A multi-dimensional plant growth regulator. Afr. J. Biotechnol. **10**: 8984-9000.

Guo B, Stiles AR and Liu CZ 2012. Thidiazuron enhances shoot organogenesis from leaf explants of *Saussureainvolucrata* Kar. et Kir. In Vitro Cell. Dev. **48**: 609-612.

Gomez, K.A., Gomez, A.A. 1984 Statistical procedures for agricultural research. Willey, New York.

Huetteman CA and Preece JE 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. **33**(2): 105-119.

Krikorian AD 1995. Hormones in tissue culture and micro-propagation. *In*: Davies PJ (ed.) Plant hormones: physiology, biochemistry and molecular biology. Kluwer, Dordrecht. pp. 774-796.

Kou Y, Ma G, Teixeira JA and Liu N 2012. Callus induction and shoot organogenesis from anther cultures of *Curcuma attenuate* Wall, Plant Cell Tiss. Organ. Cult. **112**: 1-7.

Liu JH, Zschocke S, Reininger E and Bauer R 1998. Comparison of radix angelicae pubescentis and substitutes constitutes and inhibitory effect on 5-lipoxygenase and cyclooxygenase. Pharmaceutical Biology **36**(3): 207-216.

Mallón R, Rodríguez-Oubiña J and González ML 2011. Shoot regeneration from *in vitro*-derived leaf and root explants of *Centaurea ultreiae*. Plant Cell Tiss. Organ Cult. **106**: 523-530.

Murthy BNS, Murch SJ and Saxena PK 1998.Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. In Vitro Cell Dev. Plant **34**: 267-275.

Niu XM, Li SH, Jiang B, Zhao QS and Sun HD 2002. Constituents from the roots of *Heracleum rapula* Franch. Journal of Asian Natural Products Research **4**: 33-41.

Singh CK, Raj SR, Patil VR, Jaiswal PS and Subhash 2013. Plant regeneration from leaf explants of mature sandalwood (*Santalum album* L.) trees *in vitro* conditions. In Vitro Cell Dev. Pl. **49**: 216-222.

Souri E, Farsam H, Sarkheil P and Ebadi F 2004. Antioxidant activity of some furanocoumarins isolated from *Heracleum persicum.* Pharmaceutical Biology **42**: 396-399.

Shen SC, Qian J and Ren 2010. Ethnoveterinary plant remedies used by Nu people in NW Yunnan of China. Journal of Ethnobiology and Ethnomedicine **6**: 24.

Sun HD, Lin ZW and Niu FD 1978. The study of the Chinese drugs of Umbelliferae, on the chemical constituents of the roots of *Angelica apaensis* Shan et Yuan, *Heracleum rapula* ER. and *Heracleum scabridum* FR. Acta Botanica Ainica **20**: 244-254.

Torbati M, Nazemiyeh H, Lotfipour F, Nemati M, Asnaashari S and Fathiazad F 2014.Chemical composition and *in vitro* antioxidant and antibacterial activity of *Heracleumtranscaucasicum* and *Heracleum anisactis*roots essential oil. Bio. Impacts **4**: 69-74.

Tkachenko KG 2010. Essential oils from leaves of several *Heracleum* species growing in Leningrad oblast.Chemistry of Natural Compounds **46**: 319-321.

Vasil I and Thorpe T 1994. Plant cell and tissue culture. Kluwer, Dordrecht.

Wei CC, Guan WJ, Hu DD, Zhou J and Li X 2017. Chemical constituents from *Heracleum scabridum.* Journal of Chinese Medicinal Materials **40**: 1105-1108.

(Manuscript received on 10 June, 2018; revised on 27 January, 2019)