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Clonal Propagation of 20-Hydroxyecdysone Producing Plant, *Pfaffia iresinoides*

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20-Hydroxyecdysone (20-HE) is a physiological inducer of molting and metamorphosis in arthropoda[1]. This phytoecdysteroid[2] is expected to provide progress in pest control[3], chemotherapy[4] and silk production[5]. In fact, Kozakai *et al.* demonstrated that 20-HE is an effective facilitator for the maturation of silkworms just before spinning cocoons[5].

We have obtained hairy root lines and their regenerants of *Ajuga reptans* var. *atropurpurea* with high productivity of 20-HE[6-8]. In the regenerants, however, analogs of 20-HE are also produced and they are sometimes an unfavorable factor when we intend to get only 20-HE as a pure chemical[6]. Easy isolation is important for that purpose and mainly depends on selection of plant resources that contain the target component specifically.

Pfaffia iresinoides (Amaranthaceae), a tropical plant in Brazil[9], is one of the candidates producing 20-HE predominantly. Roots of voluntary *Pfaffia* plants are currently used as the 20-HE extraction material, because the roots contain only a small quantity of analogous compounds such as polypodine B and pterosterone. Recently in Brazil, the plants have been propagated by cutting, but this propagation technique has not been sufficiently established (Mr. H. Nozawa, Central Research Com. de Agricultura Ltda., São Paulo, Brazil, personal communication).

Shoot tip culture in is an important tool for vegetative propagation of useful plants such as ornamental, woody and medicinal ones[10]. In the case of medicinal plants, not only morphological but also physiological characteristics must be stably maintained over a long culture period[10]. Schoner and Reinhard have found that the high content of cardenolide is not lost over long term culture in an excellent cultivar of *Digitalis lanata*, the plants passed through the culture show content of cardinaline equal to that cultivated in the field[11]. Hiraoka and Tomita have also found that micropropagated-clonal *Atractylodes lancea* plants show uniformly botanical and chemical evaluation[12]. In the present study, we investigated the stability of 20-HE content of a clonally propagated *P. iresinoides* plant which contains a large amount of 20-HE.

The *P. iresinoides*[9] plant was kindly given by Prof. N. Nishimoto, Tokushima Bunri University. The plant, cultivated in a greenhouse for about two years, was used as the original plant in the present study.

First of all, we measured the 20-HE content of the original plant. The 20-HE content in the plant tissues was determined as follows; 100 mg of the dried plant tissue was extracted in 5 m*l*

Table 1. Alteration of 20-HE content in clonally propagated *P. iresinoides* plants cultivated in a greenhouse or a field.

Cultivation place	Cultivation period (harvested month)	20-HE Content (% in DW) Leaf	Root
Original plants			
greenhouse	2 years (1989. 10)	0. 37	0. 42
Clonal plants*1			
greenhouse	2 months (1991. 7) *2		
	A	0. 09±0. 03*4	0. 21±0. 05*4
	B	0. 08±0. 03*4	0. 21±0. 06*4
	6 months (1991. 11) *2		
	A	0. 31±0. 12*4	0. 39±0. 11*4
	B	0. 15±0. 07*4	0. 42±0. 10*4
field	3. 5 months (1991. 9) *3		
	A	0. 46±0. 06*4	0. 35±0. 13*4
	14 months (1992. 9) *3		
	A	1. 04*5	0. 42±0. 12*4

*1 A and B indicate plant groups cultured on growth regulator-free and 1 mg/*l* NAA added MS medium for rooting, respectively.
*2 Including one-month adaptation started in June, 1991.
*3 Including one-month adaptation and one-month cultivation in the greenhouse.
*4 Mean values were obtained from measurement of three independent plants.
*5 This value was obtained from measurement of a mixture of upper, medium and lower leaves.

MeOH and 10 μl of the extract which had been passed through a membrane filter unit (column guard SJFH013NS, 0. 5 μm, Millipore Ltd.) was directly assayed by high performance liquid chromatography (HPLC: Shimazu LC-10A) using a Cosmosil 5C-18 column (25 cm in length, 4. 6 mm in inside diameter, Nacalai Tesque Inc.); flow rate was 1 m*l* min^{-1}., elution was water/acetonitrile=83/17 (v/v), and detector wavelength was 248 mm. The 2-year old original plant contained 0. 37% and 0. 42% in DW (dry weight basis) of 20-HE in the leaf and root, respectively (**Table 1**).

To establish a sterile culture of *P. iresinoides* plant, stem segments including a couple of lateral buds in each (*ca.* 2 cm in length) cut from the original plant were surface-sterilized in 70% ethanol and subsequently in 0. 5% sodium hypochlorite solution. The sterilized stem segments were cultured on 20 m*l* of Murashige-Skoog's (MS) medium[13] solidified with 0. 2% Gellan Gum (Wako Pure Pharmaceutical Inc.) in a 200 m*l*-culture flask at 25°C with a 12-hr photoperiod at 28 μmol m^{-2} sec^{-1}. from white fluorescent tubes. New plantlets emerged from the lateral buds within 5 days and rapidly grew. The stem segments including an apical bud (*ca.* 2 cm in length) cut from the plantlets were transferred to fresh MS medium for maintenance of the sterile plantlet culture every 30 days. This sterile plantlet culture was used as a material for micropropagation.

In order to establish the culture line of micropropagation, stem segments including a couple of lateral buds (*ca.* 2 cm in length) excised from the sterile plantlets were cultured on the MS medium containing either 0, 1, 2 and 5 mg/*l* benzyladenine (BA) or 0 and 1 mg/*l* naphthaleneacetic acid (NAA), or their combinations. As shown in **Fig. 1**, the addition of 1 mg/*l* BA increased the shoot number, *ca.* eight shoots with small leaves emerging from one stem segment. **Fig. 2-A** showed the general view of multiple shoots grown on MS medium with 1 mg/*l* BA. BA addition of more than 1 mg/*l* had no significant effect on increasing shoot number. NAA addition also showed no effect on increasing shoot number; rather, the cluster of shoots slightly dedifferentiated. Therefore, we decided that 1 mg/*l* BA is the suitable concentration of growth regulator available for micro-

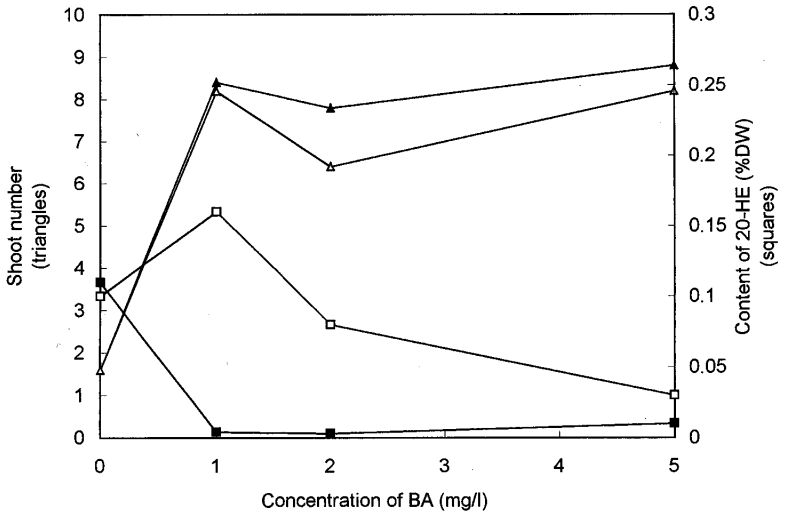

Fig. 1 Effect of BA on increasing shoot number without(▲)or with 1 mg/*l* NAA(△)and content of 20-HE without(■)or with 1 mg/*l* NAA(□)of *Pfaffia iresinoides*. The data were obtained from the average of six independent plants.

propagation of *P. iresinoides*. In this condition, the multiple shoots could actively proliferate and were maintained by transfer every 30-days. The 20-HE in the multiple shoots grown on MS medium containing more than 1 mg/*l* BA was less than 0.01% in DW(**Fig. 1**). The 20-HE content was not, however, reduced when the multiple shoots were grown on MS medium containing both 1 mg/*l* BA and 1 mg/*l* NAA(**Fig. 1**). NAA may counteract the reduction of 20-HE productivity affected by BA in the multiple shoots.

For rooting, the shoot tips(*ca.* 2 cm in length)excised from the cluster of shoots were cultured on the growth regulator-free(group A)or 1 mg/*l* NAA added MS medium(group B). **Fig. 2-B** shows the plantlets cultured on the growth regulator-free MS medium. The roots initiated on both the media but the root mass in the latter seemed to be more than that on the former(data not shown). Twelve-transferred shoots were rooted on the medium described above for adapting and cultivating in a greenhouse.

In May 1991, fifty plantlets with sufficient root mass were taken from both groups and adapted on moist vermiculite under moist conditions for a month and then were cultivated on a mixed soil (clay : sand : vermiculite=1 : 1 : 1)in pots of 10 cm in diameter for another one month in the greenhouse. Almost all the adapted plants could be cultivated in the greenhouse. As shown in **Fig. 2-C**, the cultivated plants displayed the uniformed morphology. Concerning 20-HE content, the greenhouse-grown plants demonstrated 0.08-0.09% and 0.21% DW in their leaves and roots, respectively, as listed in **Table 1**. No difference in 20-HE content between the plants belonging to each of groups was found. Twenty-HE was sufficiently accumulated in the roots of 6-month cultivated plants at the same level as that in the original plants cultivated for 2 years(0.42%). On the other hand, the contents of 20-HE in the leaves was different between group A(0.31%)and B(0.15%). The reason is unknown, but it seems that the slight differences in their cultivating conditions such as watering and illuminance between two groups influenced the amount of 20-HE.

Twenty five plants, belonging to group A, adapted and cultivated for two months in the greenhouse were transplanted to the field in September, 1991. These plants could also actively grow, but the aerial parts withered in winter. The *P. iresinoides* plant from Brazil, like other tropical plants, seems to offer a weak resistance to low temperature. In spring, however, new shoots developed

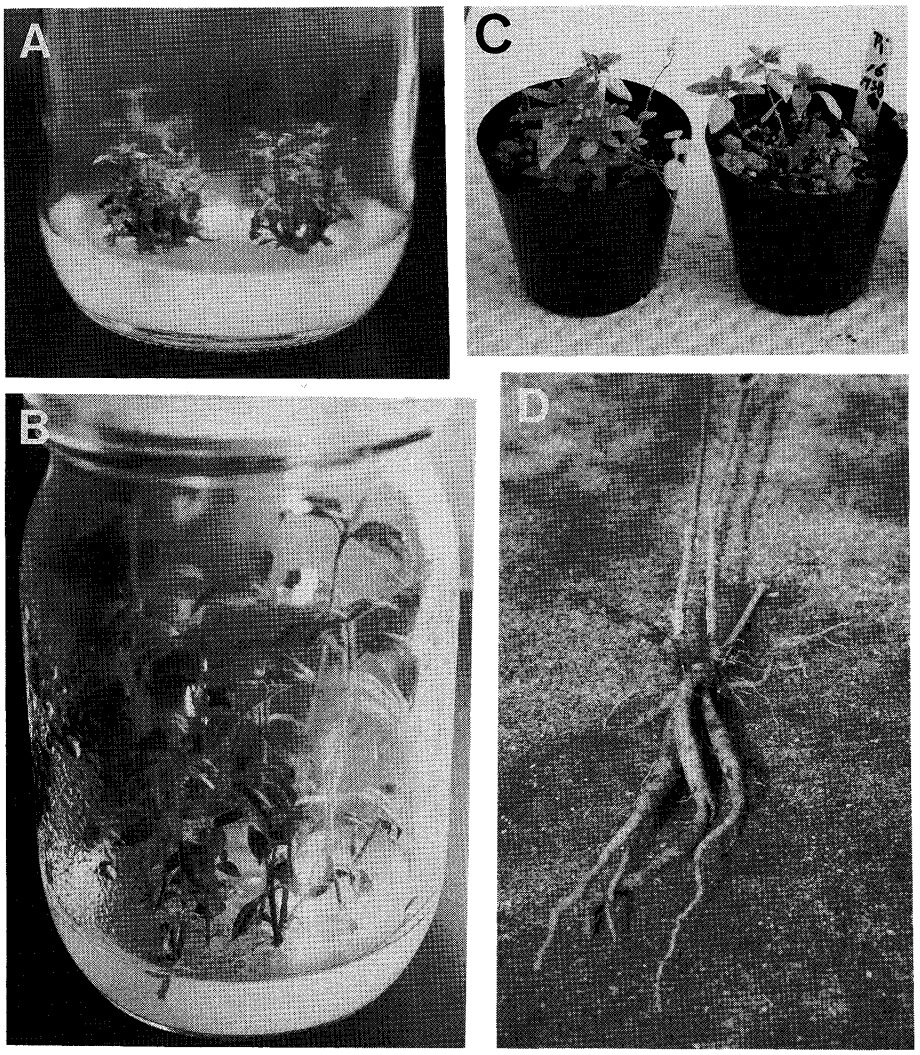

Fig. 2 Clonal propagation and cultivation of *P. iresinoides* plant.
A: Propagated shoots cultured on MS medium supplemented with 1 mg/*l* BA. B: Plants with roots cultured on growth regulator-free MS medium. C: Clonally propagated young plants cultivated in a greenhouse for a month after one-month adaptation. D: Roots of a harvested plant cultivated in the field for 12 months.

from the surviving underground roots. Twelve months after transplantation to the field (September, 1992), the plants reaching more than 2 m in height, were harvested. The roots of harvested plants were *ca.* 500 g in fresh weight. Interestingly, the root morphology of micropropagated plants was different from that of original ones; the former consists more than 5 equally branched roots elongating to 40 to 50 cm in length (**Fig. 2-D**); the latter consists of one to two primary roots elongating to 70 to 80 cm with several small secondary roots, similar to ginseng roots (data not shown). The content of 20-HE reached 1.04% and 0.42% in DW in leaf and root, respectively (**Table 1**).

The 20-HE content in the roots of clonally propagated-cultivated plant was similar to that of the original plant (**Table 1**). On the other hand, the 20-HE content in leaf is influenced by factors such as season, weather and leaf age. We found that the 20-HE content in the leaves of hybrid plants between *P. iresinoides* and *P. stenophylla* cultivated in the field had fluctuated between 0.3% and

1.0% in a year (unpublished results).

The leaves of *Pfaffia* plant are not good for extracting 20-HE, because their content of 20-HE fluctuates, their total weight per plant is smaller than that of roots, and they, moreover, contain obstacle compounds such as chlorophyll to isolating 20-HE. On the other hand, we obtained several lines of hairy roots of *P. iresinoides*[14]. It is difficult at present to maintain a constant supply of cultured hairy roots for extracting 20-HE because a large-scale culture method has not been established yet. Therefore we conclude that the root of *P. iresinoides* plant is a practical material for the extraction of 20-HE.

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《和文要約》

20-Hydroxyecdysone 生産植物 *Pfaffia iresinoides* のクローン繁殖

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ブラジル原産で，20-hydroxyecdysone 生産植物である *Pfaffia iresinoides* の茎頂を 1 mg/*l* の benzyladenine を添加した MS 培地で培養することにより，クローン繁殖系を確立することができた．生長調節剤無添加もしくは 1 mg/*l* の naphtaleneacetic acid 添加 MS 培地で発根させたクローン苗は形態が均一で，温室での馴化も容易であった．1ヶ月間温室栽培した苗，並びにさらに畑で1年間栽培した植物の 20-hydroxyecdysone 含量はいずれも約 0.4% であった．この含量は，親株のそれと類似しており，*P. iresinoides* では，クローン繁殖した植物が親株の持つ 20-hydroxyecdysone 含量を引き継ぐ可能性が示された．