

## ***In Vitro* Plant Regeneration from Leaf and Petiole Explants of *Hibiscus syriacus* L.**

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Explants from leaf and petiole of glasshouse-grown *Hibiscus syriacus* L. regenerated adventitious shoots directly or through an intermediate callus phase. Shoots were induced directly from explants cultured on Murashige & Skoog medium (MS) supplemented with 1.0–2.0 mg/l 6-benzyladenine (BA) or N<sup>6</sup>-(2-isopentenyl) adenine (2ip) and 0.1 mg/l naphthaleneacetic acid (NAA), or 2.0 mg/l BA and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) after 4 weeks of culture. Callus cultures were obtained on MS medium containing 0.2–2.0 mg/l 2,4-D and 0.2 mg/l kinetin or 0.5 mg/l zeatin. Green calli were transferred to MS medium with BA (or 2ip) – NAA, or indolebutyric acid (IBA) – gibberellic acid (GA<sub>3</sub>), where they developed adventitious shoots within 4–8 weeks of culture. About 95% shoots, either induced from the explants directly or from the callus cultures, rooted upon transferring to MS medium containing 0.5 mg/l IBA. Plants were transferred to soil and grown to flowering in the glasshouse.

### **Introduction**

*Hibiscus syriacus* L. (family Malvaceae), a perennial deciduous shrub, includes numerous cultivars having pink, purple, bluish or white flowers and is appreciated as an ornamental<sup>1)</sup>. It is also being used for the production of fibre, medicinal products and beverage additives in some Asian countries<sup>2,3)</sup>. *H. syriacus* is the hardiest species in its genus. It exhibits high resistance to pests and withstands a considerable amount of frost<sup>4)</sup>. For these reasons, it is considered as a good candidate in cell fusion experiments for the improvement of plants of the same genus, such as *H. rosa-sinensis* which is intolerant to cold<sup>5)</sup>. A few reports on *in vitro* manipulation of *Hibiscus* spp. have been published. Kenaf (*Hibiscus cannabinus* L.) callus induced from stem segment regenerated plants<sup>6)</sup>. Roselle (*H. sabdariffa* L.) callus was formed from seedling tissue<sup>7)</sup>. Callus was induced from hypocotyl section of *H. syriacus* and protoplasts were isolated from the callus<sup>8)</sup>. We have developed an efficient *in vitro* adventitious shoot regeneration system of *H. syriacus* as a part of our long-term program aimed at genetic improvement of *Hibiscus* spp. This paper reports the procedures developed to regenerate shoots from leaf and petiole explants of *H. syriacus* with or without an intermediate callus phase and regeneration of plants from the shoots.

### **Materials and Methods**

Healthy young leaves, newly developed in the spring of the year and grown to about 4 cm in size, together with the petioles were excised from glasshouse-grown *Hibiscus syriacus* cv. Heikeyama plants which were propagated by cutting in autumn of the previous year. They were surface-disinfected with 70% ethanol for 1 min. and 0.5% (v/v) sodium hypochlorite solution for 20 min., then

thoroughly rinsed three times in sterile distilled water. After disinfection, the leaves were cut into approximately 1×1 cm pieces and the petioles into 1 cm length segments.

MS medium<sup>9)</sup> containing 30 g/l sucrose, 0.8% agar, at pH 5.8 served as the basic medium (referred to as MS-B). Growth regulators of 6-benzyladenine (BA), N<sup>6</sup>(2-isopentenyl)adenine (2ip), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (KT), zeatin (ZT), naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and gibberellic acid (GA<sub>3</sub>) as shown in **Table 1** and **Fig. 2**. were used in various combinations. Both leaf and petiole explants were cultured on about 25 ml of medium in 9 cm petri dishes. All plates were incubated under 16 h light (4,000 lx with fluorescent lamp, Toshiba FL40S·D): 8 h dark photoperiod at 25°C. Each combination of growth regulator treatment was performed with at least 40 explants and each experiment was repeated twice.

After the observation of the shoot formation directly from the explants, the culture was either kept in the same medium or transferred to fresh medium with the same growth regulators as the original one for further shoot development. On the other hand, when callus was induced initially, the calli that were green in color were transferred to media indicated in **Fig. 2** and incubated until shoots appeared. Rooting of 1 cm long adventitious shoots was achieved by transferring to MS medium with 0.5 mg/l IBA.

Plantlets with well developed roots were removed from the culture vessels, washed in running tap water to remove adhering agar, planted in pots containing sterile vermiculite moistened with 0.1% HYPONeX solution (N-P-K content being 5-10-5, Murakami Sangyo, Japan) and cultured in a growth chamber in the same condition described above. After 20 days, the plants were repotted in larger pots with soil: composted leaves (3:1, v/v) and maintained in the glasshouse under natural light.

## Results and Discussion

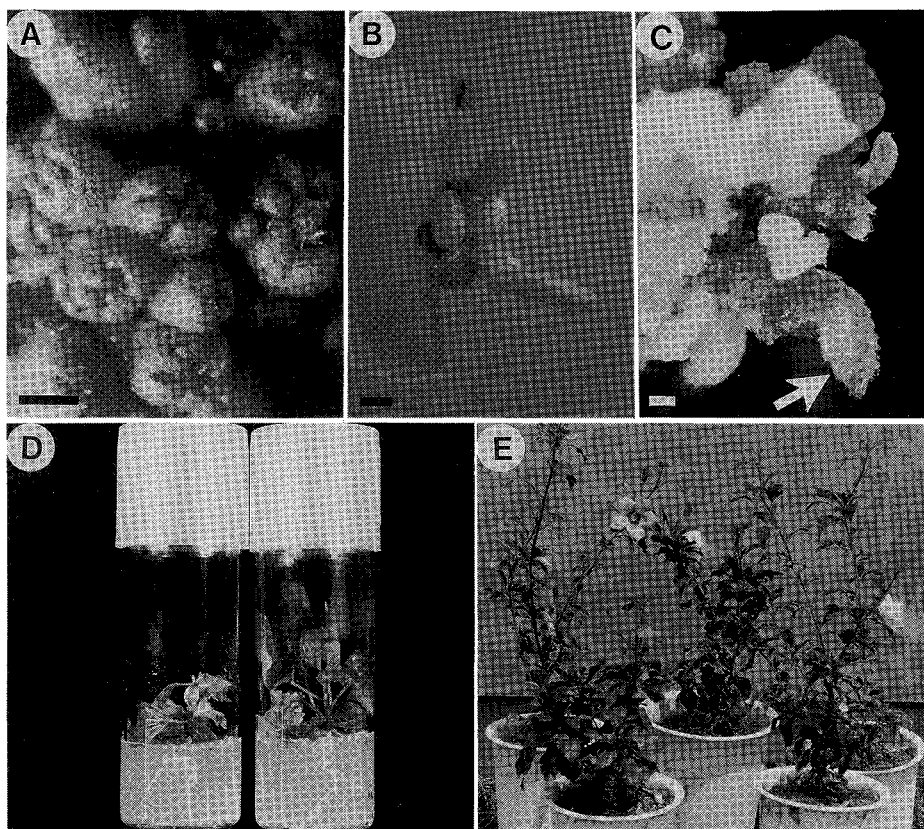
### 1. Direct shoot induction from explants

Both leaf and petiole explants of *H. syriacus* were placed on MS medium containing 8 different growth regulator combinations (**Table 1**). The explants typically showed some swelling after 5-7 days of culture. On medium 4, numerous meristematic nodular structures became visible, within 2 weeks, at the surface as well as the cut ends of the leaf and petiole explants. On medium 1, 2 and 3, however, these structures could be observed only at the cut ends of the leaf and petiole explants after 2-3 weeks of culture. In both cases, adventitious shoot buds developed from the structures after three weeks. Although numerous shoot buds were induced from the surface and the cut ends of the explants on medium 4 (**Fig. 1-A**), not all of these shoot buds could elongate and further develop into shoots of 1 cm in length. An average of 2-3 shoots per explant reached the length of up to 1 cm and further developed to normal healthy shoots. On the other hand, on the media 1, 2 and 3, only a few shoots (normally 1-3 shoots per explant) were induced at the cut ends of the explants (**Fig. 1-B**), and all of them developed into shoots up to 1 cm in length.

### 2. Callus induction and differentiation

Leaf and petiole explants, tested on medium 5, 6, 7 and 8, developed callus after 2 weeks of culture (**Table 1**). The general appearance of the calli from both type of explants was similar on media 5, 6 and 8, being friable and green in color. On Medium 7, however, the callus growth was greatly inhibited (**Table 1**), probably due to relatively high concentration of 2,4-D (2.0 mg/l). The calli induced from Medium 5, 6 and 8 would not differentiate if they were kept on the same media. We used the calli grown on Medium 5 for further shoot regeneration.

More than 20 different formulations of growth regulators were tested for callus differentiation



**Fig. 1** Shoot formation and plant regeneration from leaf and petiole explants of *Hibiscus syriacus* (bar=1 mm).

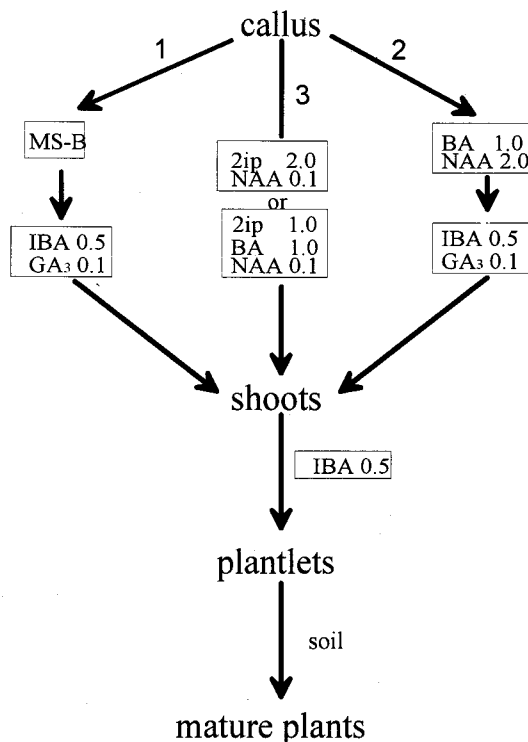
(A) Multiple shoot bud induction on Medium 4. (B) Shoot induction on medium 1, 2 and 3. (C) Shoot (indicated by an arrow) formation from callus after cultured on medium 1 or 2. (D) Root formation from shoots. (E) Regenerated plants in pots with soil showing flowering.

**Table 1.** Influence of growth regulators on callus or shoot development from leaf and petiole explants of *Hibiscus syriacus*.

Medium No.	Growth regulators(mg/l)	Callus or shoot formation and appearance from explants of	
		leaf	petiole
1	NAA (0. 1) + 2 ip (2. 0)	F	F
2	NAA (0. 1) + 2 ip (1. 0) + BA (1. 0)	F	F
3	NAA (0. 1) + BA (2. 0)	F	F
4	2, 4-D (0. 2) + BA (2. 0)	N, F	N, F
5	2, 4-D (0. 2) + KT (0. 2)	G	G
6	2, 4-D (1. 0) + KT (0. 2)	G	G
7	2, 4-D (2. 0) + KT (0. 2)	Y, P	Y, P
8	2, 4-D (0. 2) + ZT (0. 5)	G	G

The numbers in brackets represents the concentration of growth regulators.

F=1-3 shoots per explant elongated and developed, G=green callus, N= numerous shoot buds per explant initiated, P=poor callus growth, Y= yellow callus



**Fig. 2** A scheme of shoot formation and plant regeneration from callus of *H. syriacus* (the concentration of all growth regulators is in mg/l).

(data not shown). Among the formulations tested, MS medium containing 1.0 mg/l BA and 2.0 mg/l NAA (referred to as MS-BA/NAA), MS medium with 0.5 mg/l IBA and 0.1 mg/l GA<sub>3</sub> (referred to as MS-IBA/GA<sub>3</sub>), medium 1 or medium 2 were the most effective combinations for callus differentiation. There were three approaches for the production of shoot from callus (**Fig. 2**): (1) transfer of the callus, after an initial 4 weeks of culture on MS-B, to MS-IBA/GA<sub>3</sub> for additional 2 weeks; (2) growth on MS-BA/NAA for 4 weeks and transfer to MS-IBA/GA<sub>3</sub> for another 2-4 weeks. Shoot induction from callus by these two ways required a total of 6-8 weeks; (3) subculture of callus on medium 1 or medium 2 (**Table 1** and **Fig. 2**), which was the fastest way to obtain shoot from the calli. Shoots were regenerated from the calli within 4 weeks (**Fig. 1-C**).

### 3. Plant regeneration

Shoots were excised from explants or callus tissues and transferred to the vessels containing MS medium with 0.5 mg/l IBA. The shoots elongated and developed roots at a frequency of 95% (**Fig. 1-D**). When shoots were transferred to growth regulator-free MS medium, root formation was dramatically decreased to 20%.

Mature flowering plants were finally obtained from rooted plantlets which were grown in pots in a glasshouse (**Fig. 1-E**).

Our study presents a successful micro-propagation system for *Hibiscus syriacus*, a multipurpose plant resource. Various *in vitro* regeneration systems have been employed in *Agrobacterium*-mediated transformation for genetic improvement of Malvaceae plants, *e. g.* cotton<sup>10</sup> and kenaf<sup>11</sup>. The regeneration system reported here is suitable for transformation studies.

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## 《和文要約》

## ムクゲの葉及び葉柄切片からの不定芽誘導と植物体再生

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ムクゲ (*Hibiscus syriacus*) の葉と葉柄の切片からカルス及び不定芽を誘導し、再分化植物体を得た。不定芽は 1.0-2.0 mg/l の BA あるいは 2 ip と 0.1 mg/l の NAA を組み合わせた条件、または 2.0 mg/l の BA と 0.2 mg/l の 2, 4-D を含む条件の MS 培地で直接誘導された。一方、カルスは 0.2-2.0 mg/l の 2, 4-D と 0.2 mg/l の kinetin あるいは 0.5 mg/l の zeatin を組み合わせた条件で形成された。このカルスを BA (あるいは 2 ip) と NAA を、または IBA と GA<sub>3</sub> を添加した培地に移植し培養を続けたところ、不定芽が誘導された。いずれの方法で得られた不定芽も 0.5 mg/l の IBA 含有 MS 培地で培養したところ完全な植物体に再生した。