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Synthesis and antitumour activity of new derivatives of podophyllotoxin

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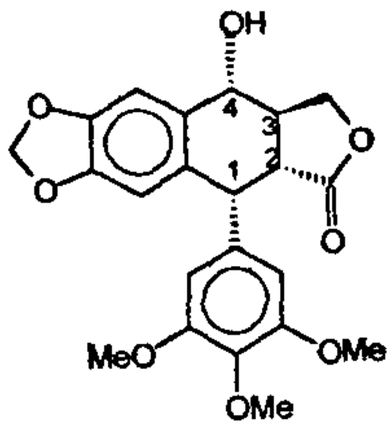
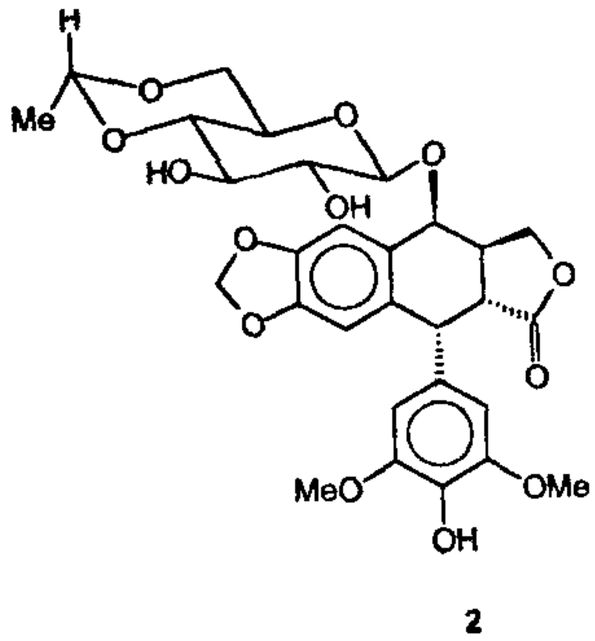
A series of new podophyllotoxin derivatives 3–8 have been synthesized and evaluated for their antitumour activity *in vitro*. Compounds 3 and 8 exhibited comparable or superior activity to clinically used etoposide (VP-16, 2) in their inhibition of human stomach carcinoma SGC-7901, lung cancer A 549, and mouse leukemia P388 cells.

SEMISYNTHETIC analogues of the naturally-occurring podophyllotoxin (**1**) have drawn much renewed interest in recent years as a result of the development of etoposide (VP-16, **2**) and teniposide (VM-26) as anticancer drugs[1,2]. It is believed that analogues of 4′-demethylepipodophyllotoxin exert their antitumour activity through stabilization of a cleavable complex between DNA and type II DNA topoisomerase. This leads ultimately to inhibition of DNA catenation activity and produces single and double strand breaks[3,4].

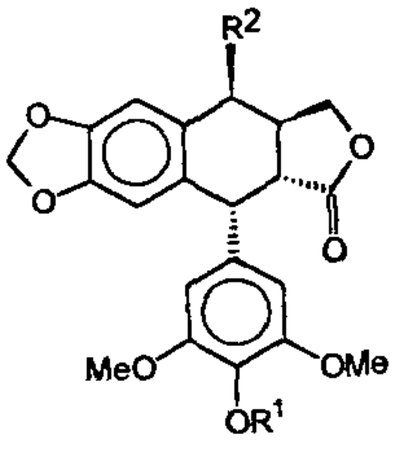
In our previous studies[5–8], we found that substitution of the glycosidic moiety in **2** by a configurationally similar nitrogen-containing group led to some compounds which have comparable or superior antitumour activity to **2**. The results suggested that the β-anomeric configuration at C-4 was indispensable for the antitumour activity. Changes in the 4β-glycosyl group are also of interest for simplified structure which might retain the activity of **2**, and be accessible to practical industrialization. Here we wish to present the synthesis of a series of new analogues of epipodophyllotoxin **3–8** and their biological activities *in vitro*.



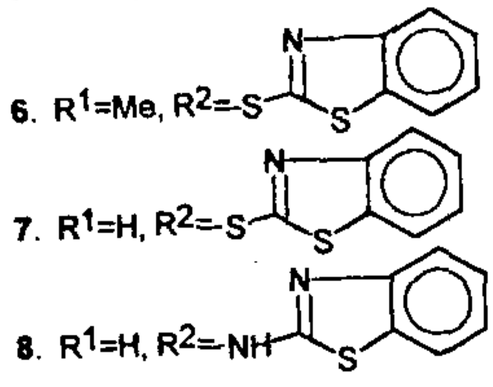
1



2



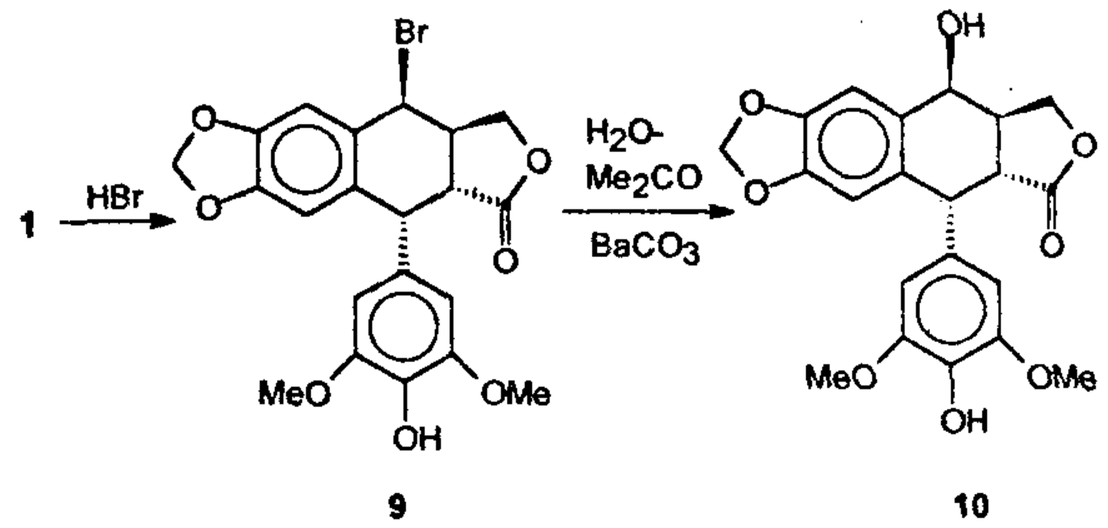
3. R^1=H, R^2=-NHCO-C_6H_4-OAc(o)
4. R^1=Me, R^2=-OCO-C_6H_4-OAc(o)
5. R^1=H, R^2=-OCO-C_6H_4-OAc(o)



Scheme 1.

The synthesis of target compounds started from **1** as shown in Scheme 2. 4β-bromo-4′-demethyl-4-deoxypodophyllotoxin (**9**) and 4′-demethylepipodophyllotoxin (**10**) were prepared from **1** by our previous procedure[5,9]. **10** was treated with HN_3 to yield 4β-azido-4′-demethyl-4-deoxypodophyllotoxin (**11**) as the major product, which was accompanied by the C-4 isomer product, 4α-azido-4′-demethyl-4-deoxypodophyllotoxin, **11** can be purified by crystallization. Further reduction of **11** led to 4β-amino-4′-demythyl-4-deoxypodophyllotoxin (**12**). Condensation of **12** with aromatic acid **13** in the presence of DCC gave compound **3**. Compounds **4** and **5** were synthesized by the reaction of **13** with **1** and **10**, respectively. Thio-etherification of **1** and **10** with 2-mercaptobenzothiazole (**14**) yielded compounds **6** and **7**, respectively. Compound **8** was synthesized by direct substitution of 2-amino-benzothiazole (**15**) with 4β-bromo-4′-demethyl-4-deoxypodophyllotoxin (**9**).

All new target compounds were characterized by m.p., ^{1}H NMR, MS and IR spectral analysis, as well as elemental analysis. The assignment of the configuration at C-4 for compounds **3–8**, **11** and **12** was based on the difference of $J_{3,4}$ coupling constants. The C-4β-substituted compounds **3–8**, **11** and **12**, have a $J_{3,4} \approx 4.0$ Hz as seen in **2** and **10** (ref. 10), due to a *cis*



Scheme 2.

Table 1. Cytotoxicity of compounds 3–8

Compound	ID50 (μM)		
	P388	SGC-7901	A549
VP-16(2)	0.012	0.32	0.44
3	0.0082	0.38	0.20
4	0.65	0.86	0.90
5	0.32	0.60	0.84
6	0.20	>10	0.40
7	0.26	>10	2.3
8	0.020	0.40	0.24

relationship between H-3 and H-4. The C-4α-substituted derivatives, however, like **1**, have a $J_{3,4} \geq 10.0$ Hz as H-3 is *trans* to H-4 (ref. 10).

We have tested the inhibitory activities of compounds **3–8** against human stomach carcinoma SGC-7901, lung cancer A549 and mouse lymphocytic leukemia P388 cells *in vitro*. As illustrated in Table 1, compounds **3** and **8** are as active or more active than VP-16 (**2**) in their inhibition of P388 and SGC-7901 cells, while compounds **3**, **6** and **8** are more potent than VP-16 in their inhibition of A 549 cells. Therefore, compounds **3** and **8** have comparable or superior antitumour activity to VP-16 (**2**). These results demonstrate the possibility of considerable simplification in the sugar structure of VP-16 and suggest further elaboration of 4β-nitrogen-containing substituent to optimize the structure of this class of anticancer compounds. Further study for anti-tumour activity of compounds **3** and **8** is in progress.

All melting points were taken on Yanaco melting point apparatus and uncorrected IR spectra were obtained on a Nicolet-5DX spectrophotometer, and ^{1}H NMR spectra were obtained by using either a Broker AM-400 or JMS-FX-90Q NMR spectrometer, all chemical shifts were reported in ppm from TMS. Elemental analyses were determined on a Carlo Elba 1106 instrument. Mass spectral analyses were taken on a V.G. ZAB-HS instrument at 70 eV with a direct inlet system.

4β-Azido-4′-demethyl-4-deoxypodophyllotoxin (**11**): To a solution of a mixture of 5.0 g (12.5 mmol) of **10** in 175 ml of anhydrous CH_2Cl_2 and 13 mmol HN_3 in benzene (10 ml) was added dropwise $BF_3.OEt_2$ (2.25 ml) at –16°C. The mixture was stirred at –16°C for 1 h. After being quenched with pyridine (2.25 ml), the solution was washed with water and dried over Na_2SO_4. Evaporation of the solvent gave a solid, which was checked by TLC and NMR analyses to be a mixture of 4α- and 4β-isomers (ca. 1:8). Crystallization from acetone/methanol provided 4β-isomer (**11**). Yield 91%, m.p. 213–214°C; MS m/z $[M]^+$ 425; IR (KBr) 3406 (OH), 2096 (azide), 1762 (lactone), 1610, 1520 and 1484 (aromatic C=C) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.82 (s, 1H, H-5), 6.58 (s, 1H, H-8), 6.27 (s, 2H, H-2′, 6′), 6.01 (d, 2H, OCH_2O), 5.47 (s, 1H, 4′-OH), 4.81 (d, J = 4.0 Hz, 1H, H-4), 4.63 (d, J = 4.4 Hz, 1H, H-1), 4.32 (d, J = 9.5 Hz, 2H, H-11), 3.78 (s, 6H, 3′, 5′-OCH_3), 3.17 (dd, J = 4.4, 12.6 Hz, 1H, H-2), 2.93 (m, 1H, H-3); Anal. calcd for $C_{21}H_{19}N_3O_7$, C59.29, H4.50, N9.88; found C59.15, H4.51, N9.77.

4β-Amino-4′-demethyl-4-deoxypodophyllotoxin (**12**): To a solution of 5.0 g (11 mmol) of **11** of 100 ml of EtOAc was added 1.0 g of 10% palladium on carbon. This mixture was shaken under 40 psi of H_2 for 16 h. The reaction mixture was filtered through celite and the filtrate evaporated *in vacuo*. Crystallization from methanol gave 3.1 g of pure **12**, yield 68%, m.p. 212–214°C; MS m/z $[M]^+$ 399; IR (KBr) 3360 (OH), 3290 (NH_2), 1745 (lactone), 1610, 1525 and 1485 (aromatic C=C) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.81 (s, 1H, H-5), 6.49 (s, 1H, H-8), 6.30 (s, 2H, H-2′, 6′), 5.96 (d, 2H, OCH_2O), 5.06 (s, 1H, 4′-OH), 4.55 (d, J = 5.2 Hz, 1H, H-1), 4.28 (d, J = 9.5 Hz, 2H, H-11), 4.20 (d, J = 4.1 Hz, 1H, H-4), 3.77 (s, 6H, 3′,5′-OCH_3), 3.28 (dd, J = 5.2, 14 Hz, 1H, H-2), 2.85 (m, 1H, H-3) ppm; Anal.

calcd for $C_{21}H_{21}O_7N \cdot H_2O$, C60.43, H5.55, N3.36, found C60.52, H5.54, N3.38.

Compound **3**: 0.20 g (0.5 mmol) of **12** was dissolved in 20 ml of dried CH_2Cl_2, 0.09 g (0.5 mmol) of acetyl salicylic acid (**13**) and 0.10 g (0.5 mmol) of DCC were added, respectively. The reaction mixture was stirred at room temperature for 5 h. Two drops of acetic acid was added. The reaction mixture was filtered. The filtrate was washed with saturated solution of $NaHCO_3$, 10% hydrochloric acid and water, respectively, dried over anhydrous Na_2SO_4, and chromatographed through silica gel using $CHCl_3$-Me_2CO (6:1) as eluent. The pure product **3** (0.14 g, 5%) was crystallized from $CHCl_3$, m.p. 214–216°C; MS m/z $[M]^+$ 561; IR (KBr) 3400–3500 (OH, NH), 1765 (lactone), 1710 (ester), 1690 (CONH), 1610, 1510 and 1480 (aromatic C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.14–7.78 (m, 4H, H-3″, 4″, 5″, 6″), 6.92 (s, 1H, H-5), 6.56 (s, 1H, H-8), 6.32 (s, 2H, H-2′, 6′), 5.99 (d, 2H, OCH_2O), 5.42 (s, 1H, 4′-OH), 4.64 (d, J = 4.0 Hz, 1H, H-4), 4.50 (d, J = 5.0 Hz, 1H, H-1), 4.20 (d, J = 9.8 Hz, 2H, H-11), 3.60 (s, 6H, 3′, 5′-OCH_3), 2.96 (m, 2H, H-2, 3), 2.21 (s, 3H, 2″-$OCOCH_3$) ppm; Anal. calcd for $C_{30}H_{27}NO_{10}$, C64.17, H4.81, N2.50, found C63.81, H4.81, N2.38.

Compound **4**: To a solution of 1.5 g (3.6 mmol) of **1** and 0.65 g (3.6 mmol) of **13** in 50 ml of dried $CHCl_3$ was added dropwise $BF_3 \cdot OEt_2$ (0.8 ml) at –16°C. The mixture was stirred at –16°C for 1 h. After quenched with pyridine (0.8 ml), the mixture was washed with water, dried over anhydrous Na_2SO_4, chromatographed on a silica gel column using $CHCl_3$-Me_2CO (7:1) as eluent, and crystallized from CH_2Cl_2 to give 1.0 g of pure product **4**. Yield 50%, m.p. 111–113°C; MS m/z $[M]^+$ 576; IR (KBr) 1778 (lactone), 1700 (ester, 1624, 1585 and 1487 (aromatic C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.21–7.91 (m, 4H, H-3″, 4″, 5″, 6″), 6.91 (s, 1H, H-5), 6.60 (s, 1H, H-8), 6.35 (s, 2H, H-2′, 6′), 6.01 (d, 2H, OCH_2O), 4.75 (d, J = 4.2 Hz, 1H, H-4), 4.42 (d, J = 5.0 Hz, 1H, H-1), 4.20 (m, 2H, H-11), 3.82 (s, 3H, 4′-OCH_3), 3.75 (s, 6H, 3′, 5′-OCH_3), 3.15 (m, 2H, H-2, 3), 2.20 (s, 3H, 2″-$OCOCH_3$) ppm; Anal. calcd for $C_{31}H_{28}O_{11}$, C64.58, H4.86, found C64.50, H4.77.

Compound **5**: **5** was synthesized from **10** by the similar procedure for the preparation of **4** from **1**. Yield 46%, m.p. 202–203°C; MS m/z $[M]^+$ 562; IR (KBr) 3367 (OH), 1778 (lactone), 1712 (ester), 1624, 1568 (aromatic C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.90 (m, 1H, H-6″), 7.60 (m, 1H, H-5″), 7.35 (m, 1H, H-4″), 7.19 (m, 1H, H-3″), 6.91 (s, 1H, H-5), 6.61 (s, 1H, H-8), 6.33 (s, 2H, H-2′, 6′), 6.01 (d, 2H, OCH_2O), 5.46 (s, 1H, 4′-OH), 4.74 (d, J = 4.3 Hz, 1H, H-4), 4.40 (d, J = 5.1 Hz, 1H, H-1), 4.15 (m, 2H, H-11), 3.61 (s, 6H, 3′, 5′-OCH_3), 3.10 (m, 2H, H-2, 3), 2.16 (s, 3H, 2″-$OCOCH_3$) ppm, Anal. calcd for $C_{31}H_{26}O_{11}$, C64.06, H4.63, found C64.27, H4.78.

Compound **6**: To a stirred mixture of 1.5 g (3.6 mmol) of **1** and 0.61 g (3.6 mmol) of 2-mercaptobenzothiazole (**14**) was added dropwise 19 ml of F_3CCOOH at –10°C. The reaction solution was stirred at room temperature for 3 h. Removal of the solvent yielded a residue, which was chromatographed on a silica gel column using $CHCl_3$-Me_2CO (7:1) as eluent, and crystallized from ethanol to obtain 0.85 g of **6**, yield 42%, m.p. 121–123°C, MS m/z $[M]^+$ 563; IR (KBr) 1772 (lactone), 1612, 1540 and 1490 (aromatic C=C) cm^{-1}; ^1HNMR ($CDCl_3$) δ 7.29–7.64 (m, 4H, H-4″, 5″, 6″, 7″), 7.00 (s, 1H, H-5), 6.58 (s, 1H, H-8), 6.38 (s, 2H, H-2′, 6′), 6.00 (d, 2H, OCH_2O), 4.67 (d, J = 4.2 Hz, 1H, H-4), 4.40 (d, J = 5.2 Hz, 1H, H-1), 4.17 (d, J = 9.5 Hz, 2H, H-11), 3.82 (s, 3H, 4′-OCH_3), 3.75 (s, 6H, 3′, 5′-OCH_3), 3.23 (m, 2H, H-2, 3) ppm; Anal. calcd for $C_{29}H_{25}NO_7S_2$, C61.81, H4.44, N2.49, found C61.58, H4.37, N2.47.

Compound **7**: It was obtained from **10** by the similar procedure for the preparation of **6** from **1**. Yield 61%, m.p. 207–209°C; MS m/z $[M]^+$ 549; IR (KBr) 3400 (OH), 1778 (lactone), 1620, 1571 and 1485 (aromatic C=C) cm^{-1}; ^1HNMR ($CDCl_3$) δ 7.28–7.83 (m, 4H, H-4″, 5″, 6″, 7″), 7.02 (s, 1H, H-5), 6.52 (s, 1H, H-8), 6.37 (s, 2H, H-2′, 6′), 5.99 (d, 2H, OCH_2O), 5.47 (s, 1H, 4′-OH), 4.66 (d, J = 4.2 Hz, 1H, H-4), 4.48 (d, J = 5.2 Hz, 1H, H-1), 4.22 (d, J = 10 Hz, 2H, H-11), 3.63 (s, 6H, 3′,5′-OCH_3), 3.20 (m, 2H, H-2, 3) ppm; Anal. calcd for $C_{28}H_{23}NO_7S_2$, C61.20, H4.17, N2.55, found C60.99, H4.15, N2.35.

Compound **8**: A mixture of 0.47 g (1 mmol) of **9**, 0.25 g (1.7 mmol) of 2-aminobenzothiazole (**15**) and 0.23 g (1.1 mmol) of $BaCO_3$ in 10 ml of $ClCH_2CH_2Cl$ was stirred at room temperature for 24 h. The reaction mixture was filtered, diluted with ethylacetate, washed with water, dried over anhydrous magnesium sulphate, and purified via silica gel column chromatography using $CHCl_3$-Me_2CO (7:1) as eluent. Crystallization from ethyl acetate provided 0.26 g of **8**, yield 49%, m.p. 218–220°C; MS m/z $[M]^+$ 532; IR (KBr) 3550–3412 (OH, NH), 1774 (lactone), 1600, 1530 and 1487 (aromatic C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.54–7.16 (m, 4H, H-4″, 5″, 6″, 7″), 6.91 (s, 1H, H-5), 6.54 (s, 1H, H-8), 6.36 (s, 2H, H-2′, 6′), 5.98 (d, 2H, OCH_2O), 5.44 (s, 1H, 4′-OH), 5.20 (br, 1H, NH), 4.59 (d, J = 4.0 Hz, 1H, H-4), 4.40 (d, J = 5.0 Hz, 1H, H-1), 4.18 (d, J = 9.5 Hz, 2H, H-11), 3.80 (s, 6H, 3′, 5′-OCH_3), 3.04 (m, 2H, H-2, 3) ppm; Anal. calcd for $C_{28}H_{24}N_2O_7S$, C63.16, H4.51, N5.26, found C62.98, H4.56, N5.17.

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High frequency somatic embryogenesis and efficient plant regeneration from hypocotyl explants of groundnut (*Arachis hypogaea* L.)

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Embryogenic calli were obtained from hypocotyl explants of groundnut (*Arachis hypogaea* L.) cultured on a medium containing different concentrations of 2,4-D or NAA in combination with 0.5 mg/l BAP. Type of auxin, concentration and genotypes influenced somatic embryogenesis. 2,4-D was found to be the best somatic embryo inductor. As the auxin level increased beyond 20 mg/l in induction medium, both per cent as well as number of embryos were decreased. 2,4-D (20 mg/l) was found to be better than NAA for the induction of globular and heart-shaped somatic embryos. Somatic embryos developed from these calli following transfer to a medium supplemented with 0.5 mg/l 2,4-D and 2.0 mg/l BAP was found to be best for embryo maturation. The well-formed embryos germinated into plantlets on MS medium supplemented with BAP (0.5–2.0 mg/l) and NAA (0.25 mg/l). The regenerated plants were transferred to soil and grown to maturity. Hardened plantlets produced normal flowers and set viable seeds. The ability of this regeneration system to produce embryos exponentially offers potential for development of new gene transfer technology and application to synseed technology.

GROUNDNUT (*Arachis hypogaea* L.) is an important grain legume and its improvement programmes include development of varieties resistant to diseases like tikka (*Cercospora arachidicola* and *Cercosporidium personatum*), rust (*Puccinia arachidis*) and pest (red hairy caterpillar). An efficient regeneration protocol either by organogenesis or somatic embryogenesis is a major prerequisite for the application of gene transfer methods for crop improvement. Regeneration by organogenesis from various explants, viz. leaves, cotyledons, cotyledonary node, hypocotyl, epicotyl, zygotic embryos has been reported[1–6]. But regeneration frequency was low and plants were rarely established. A number of recent reports describe somatic embryogenesis in peanut using a variety of different explants, including leaves[7,8], immature cotyledons[9,10] and immature embryo axes[11,12]. In spite of these studies, efficient protocol for regeneration of plantlets via somatic embryogenesis is lacking[13]. This study describes the successful induction of somatic embryogenesis from hypocotyls and their subsequent development into plants from two important commercial cultivars.

Two popular cultivars of groundnut (*Arachis hypogaea* L. cvs VRI-2 and TMV-7) were used in this investigation. Seeds were removed from the pods and immersed in 100 ml of sterile distilled water with 5 drops of Tween 80 for 5 min. Surface sterilization with 0.1% (W/V) aqueous mercuric chloride for 7–10 min was followed by 5 rinses in sterile distilled water. Seeds were germinated on MS[14] basal medium in culture tubes and kept under dark for germination at 24 ± 2°C. Hypocotyl explants of 7-day-old seedlings were used as the sources of explants.

Eight to ten hypocotyl explants (0.5–1.0 cm length) were cultured in 250 ml conical flasks. The induction medium consisted of MS salts, B5 vitamins[15], 40 g/l sucrose with different concentrations of 2,4-D or NAA (1.0, 5.0, 10, 15, 20, 25 and 30 mg/l) in combination with BAP (0.5 mg/l). The pH of the medium was adjusted to 5.8, 0.5% (W/V) agar was added, and autoclaving was done at 121°C for 15 min. The cultures were incubated for four to six weeks at 24 ± 2°C under cool-white fluorescent light at 40 $\mu E\ m^{-2}\ s^{-1}$ with a 16 h photoperiod. The per cent of embryogenesis was calculated as follows: (Number of explants showing embryogenic calli/Total number of explants cultured) × 100.