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PLANT REGENERATION AND ENHANCED TUBERIZATION IN *DIOSCOREA BULBIFERA* L. MERISTEM EXPLANTS

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ABSTRACT

A reliable and fast protocol is described for plant regeneration and enhanced tuberization in *Dioscorea bulbifera* L., a medicinally important plant of the family Dioscoreaceae. Shoot organogenesis was induced in terminal and axillary meristems within 28 days on MS, B5 and SH basal media supplemented with 0.1-1.0 mgl^{-1} benzyladenine. Rooting of shoots occurred spontaneously within 21 days on MS media in the absence of auxins, but was substantially enhanced (within 14 days) in media supplemented with 2-5 mgl^{-1} indolebutyric acid. Up to 93 per cent plantlet survival was achieved when rooted plantlets were hardened using top soil + coconut husk (1:1) as substrate, prior to transfer to potted soil in the greenhouse. Incorporation of abscisic acid at 0.02-0.20 mgl^{-1} into the planting substrate resulted in significant depression of root and shoot growth while enhancing early tuberization with consequent partitioning of assimilates in favour of tuber growth in the later growth phase.

Key Words: ***Dioscorea bulbifera*, meristem explants, enhanced tuberization.**

INTRODUCTION

Dioscorea bulbifera L. (family: Dioscoreaceae), commonly known as the aerial yam, potato yam, or bulbil-bearing yam is widely distributed throughout tropical Asia and Africa, and is the only member of the Genus *Dioscorea* that is common, in the wild state, to both continents. It also occurs in cultivation in Oceania and the West Indies (Coursey, 1967). The normal subterranean tuber is much reduced in size compared with most other yam species, and is sometimes entirely absent; even when it is sizeable, it is usually bitter, hard and unpalatable. Aerial tubers or bulbils are, however, freely produced in the leaf axils of the vine, and sometimes weighs as much as 2 kg, although 0.5 kg is a more normal weight; it is these bulbils that are used as food (Dale, 1901). Proximate analysis of *D. bulbifera* aerial tuber showed that its moisture, carbohydrate, crude protein, fat, and ash contents compare favourably with those of yellow and white yams (Igyor and Ikyo, 2004). It was also indicated that aerial yam is a good energy food source and a good substitute for white yam, especially considering the high cost of the white yam. In the best forms, these aerial tubers are said to be so succulent that they may be eaten raw, but they are more generally cooked in a similar manner to other yams, while some of the African forms require detoxification by soaking the sliced or pounded bulbils in water and prolonged boiling before being used (Burkill, 1939).

Apart from its use as food, the tubers (bulbils) serve medicinal purposes as a result of the high diosgenin (a saponin) and dioscorine (an alkaloid) contents (Coursey, 1967; Onwueme, 1978). The recent upsurge in the demand for the bulbils in traditional medicine, and as raw material for the pharmaceutical industry, coupled with its use as food, calls for improved methods of its propagation to meet these demands.

In the conventional propagation methods, the bulbils tend to form late in the annual growth cycle, when development of the stems and leaves is complete, or even when they are beginning to die away. Consequently, the period left for tuber bulking is rather short resulting in low tuber (bulbil) yield. As reported by Degras (1993), only two to three buds may be involved in *in vivo* aerial bulbil formation per plant in many

African cultivars, although Martin (1969) reported as high as eight buds for a clone (15779) from Sierra Leone.

In this paper, a complete and reliable protocol is presented for easy establishment of fast-growing shoot buds, with greater survival rate of ensuing plantlets, and enhanced tuberization in *D. bulbifera* through shoot bud culture.

MATERIALS AND METHODS

Preparation of Explants: Healthy, post-dormant aerial bulbils of *Dioscorea bulbifera* L. weighing on the average 25 - 30 g were randomly selected from accessions regularly maintained at the Botanical Garden of the Department of Botany, University of Nigeria, Nsukka, and used to raise seedlings which yielded explants employed in this study. Prior to establishment of seedlings, all the bulbils were dusted slightly with Aldrex-T (a fungicide) as protection against fungal attack. The bulbils were then sown 3-5cm deep in moistened sawdust contained in plastic pots measuring 25 cm deep and 10 cm diameter and left to sprout in a screenhouse at a temperature of 25-28 °C and 60-65 per cent relative humidity. After 42 days in the screenhouse when most of the seedlings produced bore between 8 and 12 nodes, apical (terminal) and axillary (lateral) shoot tips housing the meristems were excised and used as primary explants for this study.

Establishment of cultures: Three different basal media were employed, namely: MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and SH (Schenk and Hildebrandt, 1972) with the macro and micro salts of these media employed at full strength. Each medium was supplemented with 0-1.0 mg l^{-1} benzyl adenine (BA). Sucrose at 3 per cent (w/v) was employed as carbon source and the media were solidified with 0.8 per cent (w/v) agar (Difco-Bacto). The pH of each medium was adjusted to 5.8 before dispensing in 100 ml portions in 250 cc Erlenmeyer flasks capped with non-absorbent cotton wool wrapped in aluminium foil, prior to sterilization by autoclaving for 15 minutes at 121 °C and 103 KN M^{-2} pressure.

The explants (apical and axillary shoot tips housing the meristems) were excised singly by carefully making an incision 2-3 mm below the apical dome by means of a sterile scalpel thereby severing the shoot tip from the subtending apex (for the terminal bud) or the leaf axil (for the axillary bud). The excised explants were kept in sterile Petri dishes containing sterile distilled water prior to surface-sterilization and subsequent transfer into the appropriate media.

Explants were sterilized by dipping for 10 seconds in 70 per cent ethanol and subsequent immersion in 1.0 per cent sodium hypochlorite (NaOCl) for 10 minutes, after which they were rinsed in four changes of sterile distilled water and then inoculated onto the appropriate culture media. Three explants were inoculated per flask, with 20 replicate flasks per treatment. All transfers were done under a Laminar flow hood previously kept sterile by exposure to ultraviolet light for 30 minutes.

After the transfers, the cultures (in 250 cc Erlenmeyer flasks) were placed on racks in a growth room at 27 ± 2°C under a 14 hour photoperiod at 1,700 lux from Osram 36 W white fluorescent lamps and monitored for 28 days for shoot proliferation. Ten replicate samples were randomly selected under each treatment and scored for shoot initiation, proliferation (number produced per explant), and elongation.

Rooting of shoots and transfer of plants to soil

For rooting, explants containing shoots 3.0 – 5.0 cm high were pooled and transferred to 250 cc Erlenmeyer flasks containing full strength MS media supplemented with 0.5 - 0.3mg l^{-1} indolebutyric acid (IBA) while some of the explants were maintained in media devoid of auxins to serve as control. Each treatment consisted of 10 replicate flasks with three explants per flask. The explants were left to grow for 21 days before being scored for root formation.

Rooted explants were withdrawn from flasks and transplanted into polystyrene boxes containing the following substrates: sand + topsoil (2:1); sand + coconut husk (1:1); topsoil + coconut husk (1:1); sand + coconut husk + top soil (1:1:1). The polystyrene boxes containing the plantlets were placed in a chamber fitted with intermittent misting system in which the mist cycle was initially set for a duration of 30 seconds with a 15 minute interval in order to maintain very high relative humidity, and then lowered to one hourly interval after the first 3days in order to progressively reduce the relative humidity of the atmosphere around the plantlets. Another set or plantlets under each treatment was set up as described but without the intermittent misting in order to serve as control. The survival rate of the plants was scored after a further 14 days to evaluate the success of the acclimatization process.

Successfully hardened plantlets were then transferred singly to potted soil bathed with 0 – 0.2 mg l^{-1} abscisic acid (ABA) solution for 48 hours (to slow down vegetative growth while enhancing tuberization) prior to transfer to the greenhouse maintained at 25 – 28 °C and 60-65 per cent relative humidity to grow to maturity. At two-weekly intervals, 10 – 15 plants were randomly selected and sampled for pattern of tuberization and partitioning of assimilates to other vegetative organs (shoots and roots).

Statistical Analysis: In all the experiments, all treatments, each with 15 to 20 replicates were repeated twice. The data obtained were analyzed using ANOVA, and the means compared using Duncan's multiple range test.

RESULTS

Of the three media (MS, B5 and SH) employed in this study, MS was the most effective in inducing shoot organogenesis from the apical (terminal) and axillary (lateral) meristems in *D. bulbifera.* As shown in Table 1, the percentage of explants producing shoot buds, the number of buds produced per explant, and the number of elongated shoots per explant were all significantly higher ($P = 0.05$) under MS than under the other basal media (i.e. B5 and SH). Table 1 also shows that the addition of benzyl adenine (BA) into the medium significantly enhanced shoot regeneration. For example, while 9 %, 6 % and 5 % of apical meristem explants produced shoot buds in the absence of BA (control) under MS, B5, and SH respectively, media supplemented with BA showed a progressive increase with the highest percentages of 88 %, 63 % and 66 % recorded for 0.5 mg l^{-1} BA under MS, B5 and SH respectively. Also, while 7 %, 5 % and 6 % of axillary meristem explants produced shoot buds in the absence of BA, under MS, B5 and SH respectively, media supplemented with BA showed a progressive increase with the highest percentages of 79, 67 and 64 recorded for 0.5 mg l^{-1} BA under MS, B5 and SH respectively. This same trend was obtained for other parameters such as number of shoot buds and number of elongated shoots produced per explant (Table 1).

Rooting was induced in all the shoot explants within 14 days on media supplemented with 0.5 – 2.0 mg l^{-1} IBA (Table 2). Although roots were induced in some explants containing no auxin at all (control), the level of such induction was substantially higher in the presence of IBA at the levels tested.

The nature of the substrate in which the plantlets were acclimatized (hardened) prior to transfer to potted soil in the greenhouse significantly ($P = 0.05$) affected their survival rates (Table 3). Of the four different types of substrate employed, the highest per cent survival of plantlets was obtained in sand + coconut husk (1:1) mixture while the lowest was in sand + top soil (2:1) mixture.

Data on the time course of assimilate partitioning to the various organs (Fig. 1) showed that although there was a progressive increase in growth of all the organs with time, abscisic acid (ABA) significantly depressed shoot and root growth while enhancing tuber initiation and tuber growth at the level applied. While tuber initiation occurred by the second week in plants treated with ABA (Fig 1), it was delayed up to the 6th week in the absence of ABA(Fig. 1). By the 12th week when there was no further increase in growth of any of the organs including tuber, final tuber fresh weight values were found to have increased with increasing ABA

concentration (Fig. 2). For example, maximum tuber (bulbil) fresh weight of 201.4 ± 18.3 g was obtained under 0.20 mg l^{-1} ABA treatment as compared with its absence (control) under which tuber fresh weight of 33.7 ± 4.1g was obtained. On the contrary, the presence of ABA significantly depressed the final shoot and root fresh weights at harvest, relative to the control (Fig. 2). As percentages of the total plant weight, the highest contributions of shoot and root were 80.1 and 11.3 % respectively in the absence of ABA, and decreased with increasing ABA concentration, whereas on the contrary, the tuber contributed only 8.6 % of the total plant fresh weight in the absence of ABA, with the percentage contribution increasing with increasing ABA concentration up to a maximum of 58.7 % in the presence of 0.25 mg l^{-1} ABA (Table 4).

Table 1: Effect of benzyl adenine (BA) on shoot regeneration from apical and axillary meristem explants of *Dioscorea bulbifera* after 28 days of incubation in three different standard basal media (MS, B5 and SH)

Treatment		Apical meristem			Axiliary Meristem		
Basal medium	BA conc. (mgl^{-1})	% Explants producing shoot buds	No. Shoot Buds per explant	No. Elongated shoots per explant	% Explants producing shoot Buds	No. shoot buds per explant	No. elongated shoots per explant
MS	0.0	9	0.2±0.1	0±0	7	0.2±0.1	0±0
	0.1	69	2.2±0.9	1.4±0.3	63	1.9±0.6	1.0±0.3
	0.2	73	3.1±0.8	2.3±0.7	69	2.6±0.6	1.9±0.6
	0.5	88	4.2±0.9	3.8±0.5	79	3.9±0.8	3.3±0.8
	1.0	49	1.3±0.5	0.9±0.2	43	1.4±0.6	0.7±0.3
B5	0.0	6	0.1±0.1	0±0	5	0.1±0.1	0±0
	0.1	53	0.7±0.2	0.5±0.1	55	0.4±0.1	0.6±0.2
	0.2	57	1.0±0.4	1.1±0.3	58	1.3±0.6	0.8±0.3
	0.5	63	2.7±0.7	1.9±0.5	67	2.4±0.8	2.4±0.7
	1.0	42	1.7±0.6	0.7±0.3	40	1.1±0.3	0.5±0.1
SH	0.0	5	0.3±0.1	0±0	6	0.2±0.1	0±0
	0.1	49	0.5±0.2	0.3±0.1	55	0.3±0.1	0.4±0.1
	0.2	59	1.2±0.3	0.9±0.3	57	1.3±0.7	0.7±0.2
	0.5	66	2.5±0.8	1.7±0.6	64	2.0±0.9	2.1±0.7
	1.0	37	1.8±0.7	0.8±0.4	44	1.3±0.7	0.3±0.1

Table 2: Rooting of *D. bulbifera* shoots arising from 0.5mgl^{-1} BA-treated meristem explants as affected by IBA treatments

IBA conc. (mgl^{-1})	Per cent explants producing roots	Number of roots per explant	Length (cm) of roots per explant	Fresh weight (mg) of roots per explant
0.0	43	1.8±0.6	0.9±0.3	19.9±2.2
0.5	100	4.8±1.6	3.3±1.7	29.2±6.2
1.0	100	5.3±1.9	3.9±1.6	37.8±5.7
1.5	100	4.2±1.3	3.3±1.2	37.4±5.3
2.0	100	4.4±1.3	3.7±1.4	40.7±6.6
2.5	79	2.8±0.9	3.5±1.1	34.4±4.7
3.0	54	2.3±0.6	2.0±0.6	22.5±3.1

Table 3: Effect of different planting substrates on the survival rate of *D. bulbifera* plantlets arising from shoot explants rooted in 0 - 3.0 mgl^{-1} IBA

	Survival of *in vitro* plantlets in different substrate combinations			
IBA conc. (mgl^{-1}) in rooting medium	Sand + Top soil (2:1)	Sand + coconut husk (1:1)	Top soil + coconut husk (1:1)	Top soil +coconut husk + sand (1:1:1)
0.0	59	91	66	74
0.5	64	93	64	77
1.0	61	92	61	80
1.5	63	89	57	69
2.0	59	88	61	76
2.5	85	89	62	77
3.0	61	83	59	69

Table 4: Percentage contribution of individual organs to total plant weight at harvest of *D. bulbifera* plants raised from ABA-treated shoot explants under greenhouse conditions

	Relative assimilate contribution (%)			
ABA concentration (mgl^{-1})	Shoot	Root	Tuber	Total
0.0 (control)	80.1	11.3	8.6	100
0.02	71.4	9.9	18.7	100
0.05	67.7	9.4	22.9	100
0.08	62.8	8.9	28.3	100
0.10	48.6	10.3	41.1	100
0.15	41.8	9.1	52.9	100
0.20	32.9	8.9	58.2	100
0.25	38.1	8.2	58.7	100

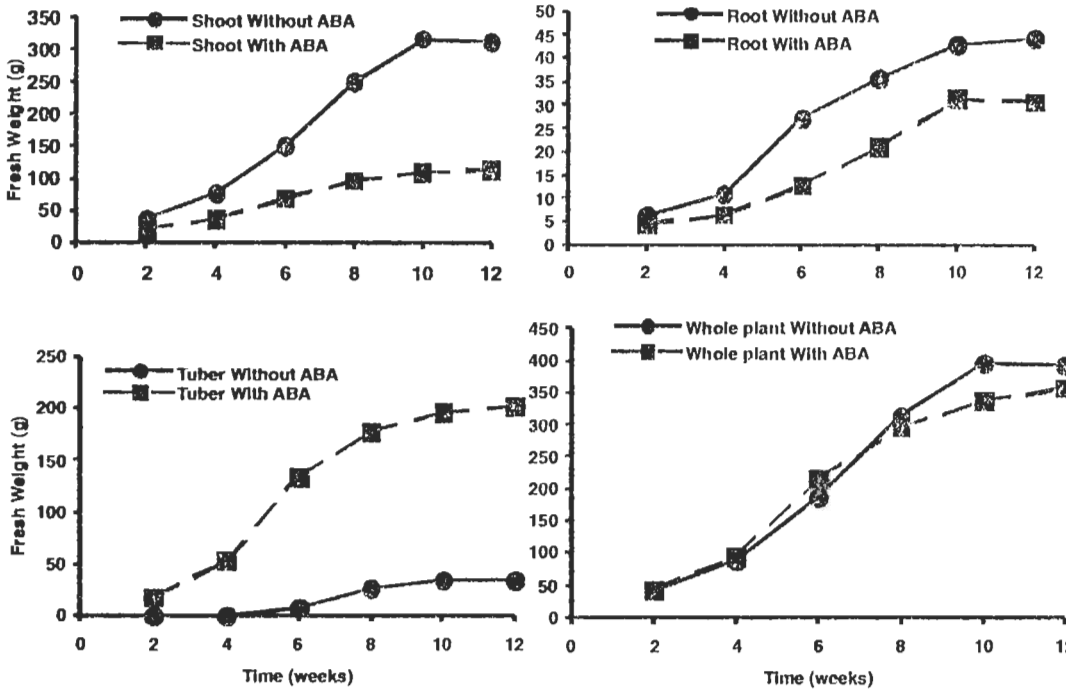

Fig. 1: Time course in distribution of assimilates to the various organs and tuber in *D. bulbifera* pla during a twelve week growing period, as affected by 0.2 mgl^{-1} ABA treatment.

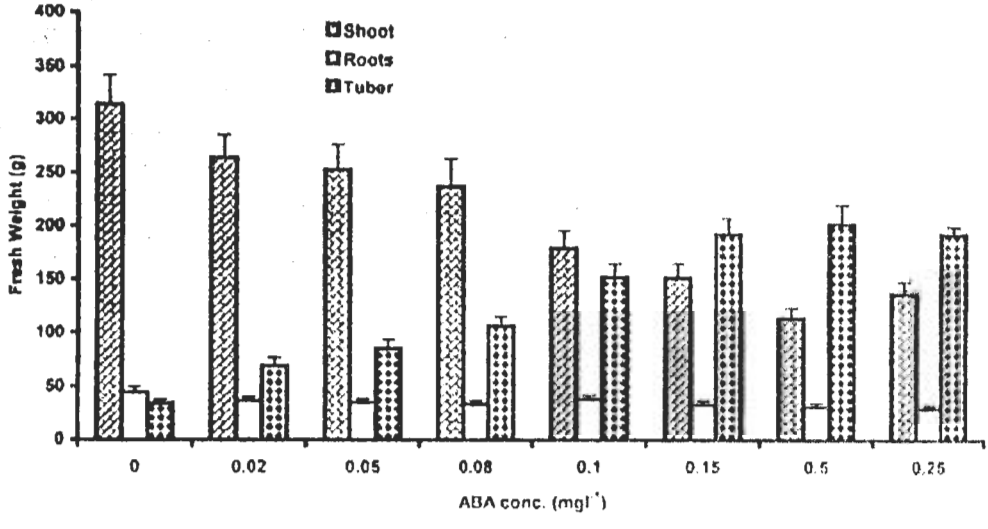

Fig. 2: Fresh weight of vegetative organs (shoot and roots) and tuber (bulbils) produced at harvest from bulbifera plants raised from bud explants as affected by abscisic acid (ABA) treatment

DISCUSSION

Of all the growth parameters considered in this study, namely; per cent of explants producing shoot buds, number of shoot buds per explant, and number of elongated shoots per explant, MS was superior to both B5 and SH in shoot organogenesis in *D. bulbifera*. A close examination of the ion content of each of these three media shows that although each of them contains most of the macro and micro elements considered to be essential in media formulation for *in vitro* organogenesis, MS differs from the rest in its higher nitrogen content in the form of NH_4^+, together with the use of higher concentrations of NO_3^-, and this might have contributed to its superiority to the other two media (B5 and SH) in permitting substantially greater shoot organogenesis. These findings are consistent with the report by George and Sherrington (1984) that the introduction of nitrogen in the form of NH_4^+, together with the use of higher concentrations of NO_3^- and K^+ in the MS medium, permitted better direct and indirect shoot formation and embryogenesis in a very wide range of tissue cultures. Similar findings have also been reported for *Typhonium flagelliforme* bud explants (Sai *et al*., 2000).

Irrespective of the basal medium type, this study showed that shoot formation in *D. bulbifera* terminal and axillary meristem explants is strongly dependent on the presence of BA in the medium. This is demonstrated by the fact that while only 9, 6 and 5 per cent of apical meristem explants, and 7, 5 and 6 per cent of axillary meristem explants produced shoots in MS, B5 and SH media in the absence of a cytokinin (BA), up to 88, 63 and 66 per cent of apical meristem explants, and 79, 67 and 64 per cent of axillary meristem explants were produced in the presence of 0.5 mg l^{-1} BA in MS, B5 and SH respectively. Similar trends have also been reported for shoot organogenesis in many other species such as *Boswellia serrata* (Purohit *et al*., 1995) and from cotyledonary node explants of *Pterocarpus marsupium* (Chand and Singh, 2004).

While shoot buds arising from 0.5 mg l^{-1} BA-treated explants produced roots spontaneously within 14 days in the absence of an auxin (IBA), those cultured in MS media supplemented with 0.5 – 2.0 mg l^{-1} IBA recorded 100 per cent rooting within the same period, indicating the need for an auxin in root formation in these (bud) explants. That there was up to 43 per cent rooting in the absence of IBA must have been due to the presence of some level of a native auxin (albeit lower in concentration than 0.5 mg l^{-1}) within the cultured shoot buds. External application of auxins has been shown to enhance root formation within 21 days in an earlier study with nodal explants of *D. bulbifera* (Okezie, 2001) and cotyledonary node explants of *Pterocarpus marsupium* (Chand and Singh, 2004).

The shoot or root regeneration medium in which the explants were raised notwithstanding, survival rate of the explants prior to transfer to potted plants in the greenhouse or field is dependent upon both the humidity regime during the hardening process and the nature of the substrate. The drastically reduced survival rate (0 – 3 per cent) of plantlets maintained in an environment with ambient relative humidity as compared with those maintained under intermittent misting (58 – 93 per cent survival) was certainly as a result of the drastic drop in relative humidity of the former as they moved from *in vitro* to *ex vitro* conditions. It has been pointed out by George and Sherrington (1984), Pierik (1987) and Kumar *et al.* (2004) that plantlets in culture tubes are adapted to high relative humidity (almost 100 %) and it is, therefore, important that an environment with high relative humidity be provided. *In vitro* plantlets usually have poorly developed cuticle, the stomata are not functioning properly and the vascular development between root and shoot may not be complete. These restrict water transport while at the same increase water loss (Ziv, 1979).

Of all the four substrates employed during hardening, namely: sand + topsoil (2:1), sand + coconut husk (1:1), topsoil + coconut husk (1:1), and topsoil + coconut husk + sand (1:1:1), sand and coconut husk (1:1) was found to be the best substrate for transferring *in vitro* plantlets, with up to 93% survival rate. Sand + topsoil (2:1) and coconut husk + topsoil (1:1) reduced the survival rates of the plantlets (59 and 57 per cent respectively). As reported by Evans (1990), and Sai *et al.* (2000), for successful transfer of plantlets to the

field, high humidity must be maintained, and the planting substrate must not be waterlogged. The topsoil which had high water-holding capacity had caused the substrate to be waterlogged resulting in some of the plantlets rotting, and hence reduced the survival rate.

Partitioning of assimilates in favour of tuberization (initiation and subsequent development) at the expense of shoot and root growth in the presence of abscisic acid (0.2 mgl^{-1}), as found in this study, has positive implications for the propagation of this medicinally important tuberous crop since the economic yield (both as food for humans and medicinal purposes) of *D. bulbifera* is mostly the tuber (Coursey, 1967; Degras, 1993). Methods described in this study in which tuber fresh weight of 201.4 g was obtained at harvest in the presence of 0.1mg l^{-1} abscisic acid ensure greater tuber yield than those in earlier studies on the same crop (Okezie, 2003) in which a maximum tuber yield of 164.6 g was obtained under the same conditions. The use of growth retardants within the same concentration threshold for the enhancement of tuberization has been reported for some other tuberous crops such as Irish potato, *Solanum tuberosum* (Powell and Caligary, 1989) and sweet potato, *Ipomoea batatas* (Liu *et al.*, 2001).

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