In vitro regeneration of *Quercus floribunda* Lindl. through cotyledonary nodes: an important tree of Central Himalaya

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A regeneration protocol has been developed for *Quercus floribunda* Lindl. using cotyledonary nodes (with attached cotyledons but without radicle and primary shoot) as explants. Multiple shoots were induced on woody plant (WP) or MS medium supplemented with 6-benzyladenine (BA), either alone or in combination with gibberellic acid (GA_3). BA (22.19 μM) was much more effective in WP medium for induction of multiple shoots; addition of GA_3 (2.89 μM) resulted in thinner but slightly longer shoots. Rooting (83.3%) of regenerated shoots involved a two-step procedure where the microshoots were treated with indole-3-butyric acid (100 μM) for 24 h followed by transfer to plant growth regulator-free half-strength WP medium. Ninety per cent plantlets were successfully established in earthen pots containing soil and farmyard manure (3 : 1).

A total of 450 species of *Quercus* are distributed throughout the temperate regions of the world[1]. Five evergreen species, namely *Q. glauca* (phaniyat oak), *Q. leucotrichophora* (banj oak), *Q. floribunda* (tilonj oak), *Q. lanuginosa* (rianj oak) and *Q. semecarpifolia* (brown oak) occur in the Central Himalaya between 1000 and 3600 m amsl (ref. 2) and represent the climax vegetation. They have assumed considerable relevance not only for their economic use but also for the general health of the fragile ecosystem. The regeneration of oak species in the region is reported to be steadily deteriorating[2] due to (i) excessive lopping, (ii) irregular fructification and unavailability of mass seed crop every year, (iii) low acorn production and long reproductive cycle, (iv) seed infestation by various pests in nature as well as during storage, (v) consumption of seeds by animals and birds, and (vi) short viability of seeds. Further, clonal (vegetative) propagation of oaks through stem cuttings has not been very successful[3,4].

Tissue culture could be useful in overcoming some of the above-cited difficulties[5] and to multiply existing 'elite' germplasm. Although *in vitro* propagation via axillary shoot multiplication has been reported for some oak species, namely, *Q. robur*, *Q. petraea*[6–9], *Q. suber*[10–12], *Q. shumardii*[13], *Q. acutissima*[14], *Q. serrata*[15], *Q. rubra*[16], *Q. glauca* and *Q. leucotrichophora*[17], micropropagation of *Q. floribunda* has not been reported. *Q. floribunda* (an important local species) was selected for the development of *in vitro* micropropagation protocol through multiple shoot formation using cotyledonary nodes as initial explants.

Seeds of *Q. floribunda*, collected from the forest at Kilbury (2100 m amsl), Nainital were separated from the cupule, wetted in a detergent solution (Labolene, 0.1%, v/v; 10 min), and washed under running tap water for 5 min. Subsequently, the seeds were rinsed in distilled water (× 4), sequentially treated in solutions containing a systemic fungicide (Bavistin, 0.2%, w/v; 30 min), an antioxidant (ascorbic acid, 0.02%, w/v; 30 min), and finally surface-disinfected with an aqueous solution of mercuric chloride (0.05%, w/v; 10 min). Each treatment was followed by repeated washings (× 4) with sterile distilled water.

After removing the coat from disinfected seeds, the seeds were inoculated on agar gel (0.8%, w/v) containing only sucrose (3.0%, w/v). Seeds started germinating and the cotyledons became greenish within eight days after inoculation. After two weeks the radicle and primary shoot were removed from the germinating seeds, and the cotyledonary nodes (3–4 per culture flask) were transferred to Murashige and Skoog (MS) or woody plant (WP) medium containing sucrose (3.0%, w/v) and agar (0.8%, w/v) (pH adjusted to 5.8; 100 ml medium in 250 ml Erlenmeyer flasks, autoclaved at 1.05 kg/cm^2 pressure at 121°C for 20 min), and supplemented with various concentrations of 6-benzyladenine (BA, 2.22–22.19 μM) and gibberellic acid (GA_3, 2.89 μM). One set of explants was cultured on medium without plant growth regulators (PGRs) that served as control. Each treatment consisted of 24 explants and all experiments were repeated at least once. The cultures were maintained at 25 ± 1°C in a 16 h light and 8 h dark cycle, with irradiance (42 μmol m^{-2} s^{-1}) by cool fluorescent tubes (Philips, 40 W). Subculturing was generally carried out at four-weeks interval and data on shoot number and shoot length were recorded after 30 days of inoculation. For continued production of shoots, subculturing of cotyledonary nodes was carried out on WP medium supplemented with 22.19 μM BA, which gave better response in comparison to MS medium. This was continued up to the fourth subculture and data on the number of shoots formed during each subculture, shoot height and shoot diameter were recorded (details not shown).

For root induction, microshoots (2.0–3.0 cm height with 2 or 3 leaflets) were transferred to half-strength WP medium containing sucrose (3.0%; w/v) and phytagel (0.25%; w/v), and supplemented with indole-3-butyric acid (IBA; 0.44, 2.46, 4.92, 7.38, 9.84, 14.76 and 24.61 μM). While rooting was good, this also resulted in basal callus formation in some shoots. To overcome this problem, the

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excised microshoots were treated with IBA (25–100 µM) for 24 or 48 h only, and then transferred to PGR-free half-strength WP medium. The shoots with well-developed roots were taken out from the culture medium after 25 days. The roots were gently washed with water to remove traces of phytagel and the plantlets were transferred to small plastic cups (6.1 cm diameter, 8.5 cm height) containing garden soil and farmyard manure (3 : 1, v/v). These were kept in a mist chamber (25°C, 80% RH) for acclimatization.

One-month-old acclimatized plants were transferred to earthen pots (21 cm diameter, 22 cm height) containing garden soil and farmyard manure (3 : 1; v/v). Following transfer to pots, growth performance and per cent survival were recorded at three months interval. After six months, the acclimatized plants were shifted to nursery conditions with partial shade for the first ten days and then moved to a place where the seedlings received full sunlight.

Statistical analyses were done following published methods[18,19].

Culture of excised cotyledonary nodes (size approx. 2 cm, with two intact and attached cotyledons; Figure 1 *a*) obtained from germinated seeds on MS or WP medium, supplemented with various concentrations of BA and GA_3 resulted in shoot formation. However, the number of shoots formed varied with the treatment (Table 1). The results of ANOVA indicate that the number of shoots significantly differed ($P \leq 0.01$) with respect to the medium and PGR concentration. The highest concentration (22.19 µM) of BA, when used alone, was found to be effective, and the average number of shoots per cotyledonary node increased to 4.0 with average shoot length of 1.8 cm on MS medium and 11.0 shoots with average shoot length of 3.9 cm on WP medium (Figure 1 *b*). However, addition of GA_3 (2.89 µM) together with BA was less effective and resulted in thinner but slightly longer shoots (Table 1). It was, therefore, omitted from

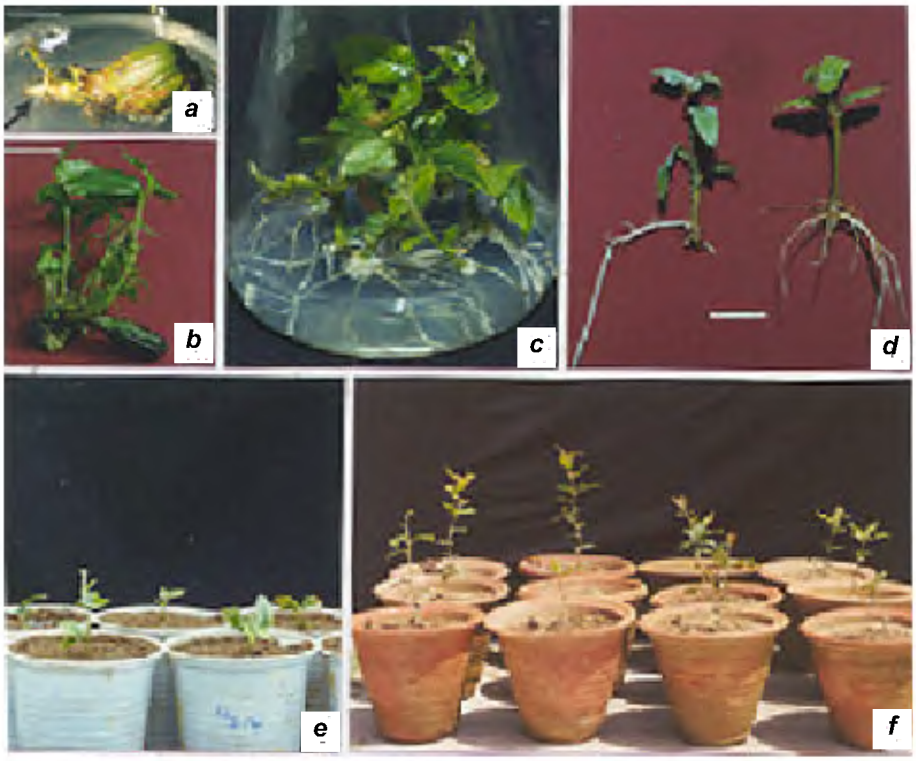

Figure 1. ***a***, Original explant (decoated seed) of *Quercus floribunda* was kept on agar gel medium for two weeks. Following germination, the primary shoot (white arrow) and radicle (black arrow) were excised and the cotyledonary nodes were transferred to WP medium supplemented with BA and GA_3 for production of multiple shoots. This picture was taken two weeks after transfer of cotyledonary nodes to WP medium. Bar indicates 1.2 cm; ***b***, Multiple shoot formation from cotyledonary nodes of *Q. floribunda* 30 days after transfer on WP medium supplemented with 22.19 µM BA. Bar indicates 1.5 cm; ***c***, Rooting of microshoots of *Q. floribunda* after three weeks of transfer on half-strength WP medium in the continuous presence of 14.76 µM IBA. Note the presence of callus at the base of some well-rooted shoots; ***d***, Rooted microshoots of *Q. floribunda* taken out after 25 days on PGR-free half-strength WP medium following an initial exposure to 100 µM IBA for 24 h. Bar indicates 1 cm; ***e***, *In vitro*-raised plants of *Q. floribunda* one month after transfer to plastic cups just before transfer to earthen pots, and ***f***, Well-established plants in earthen pots nine months following transfer to soil (one month in plastic cups followed by eight months in earthen pots).

the final protocol. Shoot multiplication increased when the main shoot was excised, possibly due to reduced apical dominance. Similar results have also been reported for rose[20] and for *Q. glauca* and *Q. leucotrichophora*[17]. BA-induced axillary shoot proliferation from the cotyledonary nodes of seedlings has also been reported in *Alnus glutinosa*[21]. A similar pattern was also reported in apple[22], where the nodal explants produced more shoots than the apical explants. BA at 22.19 µM induced multiple shoot buds in all explants within a week. The buds appeared as small green protuberances on the cotyledonary nodes that elongated into shoots possessing leaves. Following the harvest of the first crop of shoots for rooting, cotyledonary nodes could be cultured again for the growth of further shoots. The number of shoots, however, decreased after repeated subculture of cotyledonary nodes on the same medium (Table 2). After the fourth subculture the values decreased to a minimum of 1.3 shoots, with an average shoot length of 1.2 cm (Table 2). Following the harvest of this crop of shoots from the cotyledonary nodes, no more shoots appeared on further culture of by now the badly shrivelled cotyledonary nodes.

Within 30 days of subculturing on WP medium, up to 11 shoots per cotyledonary node could be obtained. This is several times higher, in comparison to 2–3 shoots per nodal explant reported for *Q. robur*[8]. BA stimulated higher shoot multiplication in comparison to other cytokinins tested in *Q. robur*[8,23]. Further, BA has been reported to significantly influence *in vitro* growth and proliferation of shoots in *Q. robur*[6,7]. It is also recorded to be the best inducer of shoot multiplication in *Q. shumardii*[13]. It may be mentioned that BA, its riboside and nucleotides have also been reported as naturally occurring cytokinins in plant tissues[24] and are relatively stable in comparison with other cytokinins[25]. This may explain the improved response with BA.

Table 1. Effect of medium and plant growth regulators on multiple shoot formation in cotyledonary nodes of *Q. floribunda* (± SE)

PGR[a] treatment (µM)		MS			WP		
BA	GA_3	Number of shoots/ explant	Avg. length of shoots (cm)	Avg. length of longest shoots[b] (cm)	Number of shoots/ explant	Avg. length of shoots (cm)	Avg. length of longest shoots[b] (cm)
0.0	0.0	2.0 ± 0.47	3.0 ± 0.19	3.5 ± 0.23	1.3 ± 0.27	5.2 ± 0.72	5.4 ± 0.67
2.22	0.0	3.3 ± 0.72	3.6 ± 0.18	5.1 ± 0.05	3.3 ± 0.72	3.1 ± 0.10	4.3 ± 0.95
4.44	0.0	3.0 ± 0.47	2.4 ± 0.30	3.7 ± 0.59	4.7 ± 0.98	2.3 ± 0.20	3.8 ± 0.36
22.19	0.0	4.0 ± 0.47	1.8 ± 0.26	3.8 ± 0.52	11.0 ± 0.47	3.9 ± 0.40	5.1 ± 1.20
2.22	2.89	2.7 ± 0.27	4.1 ± 0.61	5.6 ± 0.94	5.3 ± 0.72	3.8 ± 0.85	5.3 ± 0.89
4.44	2.89	3.7 ± 0.27	2.7 ± 0.27	4.0 ± 0.40	6.3 ± 0.72	3.3 ± 0.02	4.3 ± 0.36
22.19	2.89	4.0 ± 0.81	3.4 ± 0.60	5.5 ± 1.43	3.6 ± 0.27	3.6 ± 0.45	5.8 ± 1.34
LSD ($P \leq 0.05$)		1.99	1.44	2.73	2.50	2.28	3.34

ANOVA summary table

Source	DF	Mean square	*F*-ratio
Shoot number			
PGR concentration	6	16.21	9.59*
Media type	1	29.16	17.25*
PGR × media type	6	6.27	3.71**
Error	26	1.69	
Avg. length of longest shoot			
PGR concentration	6	2.86	1.10 ns
Media type	1	1.60	0.61 ns
PGR × media type	6	2.04	0.78 ns
Error	26	2.59	
Average shoot length			
PGR concentration	6	2.88	2.52**
Media type	1	1.28	1.12 ns
PGR × media type	6	1.76	1.54 ns
Error	26	1.14	

*Significant at 0.01 level; **Significant at 0.05 level; ns: Not significant.
[a]PGR, Plant growth regulator; [b]Values are an average of longest shoots in each of the eight flasks.
MS, Murashige and Skoog medium; WP, Woody plant medium; SE, Standard error. Data were recorded 30 days after inoculation. All values are an average of 24 explants (eight flasks with three explants per flask) and the experiment was repeated twice with similar results.

Table 2. Effect of subculturing on shoot multiplication in cotyledonary nodes of *Q. floribunda* on WP medium supplemented with 22.19 µM BA (± SE)

Culture	No. of shoots/explant	Avg. shoot length (cm)	Avg. length of longest shoots* (cm)
Initial	11.0 ± 0.47	3.9 ± 0.40	5.1 ± 1.20
1st subculture	5.3 ± 0.19	1.7 ± 0.21	3.3 ± 0.40
2nd subculture	4.9 ± 0.58	2.0 ± 0.12	3.1 ± 0.13
3rd subculture	3.0 ± 0.07	1.4 ± 0.10	2.1 ± 0.15
4th subculture	1.3 ± 0.27	1.2 ± 0.72	1.4 ± 0.67

Data are an average of 24 explants (eight flasks with three explants per flask). The experiment was repeated once.
*Values are an average of longest shoots in each of the eight flasks.

Table 3. Effect of short treatment with IBA on rooting of *Q. floribunda* microshoots (± SE) following transfer to PGR-free half-strength WP medium

Treatment with IBA (µM)	Time (h)	Per cent rooting	Avg. no. of roots per shoot	Avg. length of roots (cm)
0.0[a]	24	0.0	0.0	0.0
	48	0.0	0.0	0.0
25.0	24	0.0	0.0	0.0
	48	0.0	0.0	0.0
50.0	24	50.0	2.3 ± 0.27	1.5 ± 0.31
	48	33.3	2.5 ± 0.35	1.7 ± 0.17
75.0	24	66.6	2.5 ± 0.55	1.8 ± 0.38
	48	33.3	2.5 ± 0.35	1.0 ± 0.17
100.0	24	83.3	3.6 ± 0.21	1.6 ± 0.30
	48	50.0	2.6 ± 0.27	2.1 ± 0.12
LSD ($P \leq 0.05$)			0.41	0.33

[a]Shoots treated with sterilized milli Q water for 24 or 48 h. Data are an average of 20 microshoots. The experiment was repeated once.

Table 4. Establishment of *in vitro*-raised plants of *Q. floribunda* after transfer to soil (± SE)

Month	Per cent survival	Plant height (cm)	No. of leaves	Shoot diameter (mm)	No. of nodes
0[a]	100	2.0 ± 0.18	2.2 ± 0.49	0.7 ± 0.05	1.2 ± 0.19
3	90	5.2 ± 0.72	5.9 ± 1.03	1.4 ± 0.15	2.3 ± 0.30
6	90	10.5 ± 1.22	7.3 ± 1.40	1.7 ± 0.20	3.3 ± 0.38
9	90	18.7 ± 2.02	8.2 ± 1.52	2.2 ± 0.25	4.8 ± 0.54
LSD ($P \leq 0.05$)		4.9	4.2	0.7	1.30

[a]Day of transfer to soil. Diameter was recorded 1 cm above the ground. Data are an average of 20 plantlets.

Half-strength PGR-free WP medium failed to induce *in vitro* rooting of microshoots (average height 2.0–3.0 cm, with 2 or 3 leaflets) even after 25 days of culture (Table 3). The excised microshoots, when treated with different concentrations of IBA for a brief period and then placed in PGR-free half-strength WP medium with phytagel (0.25%) and sucrose (3.0%) exhibited up to 83.3% rooting (Table 3). While continuous exposure to IBA (results not shown) also resulted in good rooting of microshoots, formation of callus at the base was noticed in some cases (Figure 1 *c*). The best results were achieved when microshoots were treated with 100 µM IBA for 24 h and thereafter placed in PGR-free half-strength WP medium. In this process around 80% shoots were successfully rooted without the formation of basal callus (Table 3, Figure 1 *d*). This two-step method of rooting is also found to be effective in *Q. suber*[12], *Thamnocalamus spathiflorus*[26], *Q. glauca*[17] and *Q. leucotrichophora*[17]. Although auxins are essential for root induction, these may not be required for root growth; rooting is sometimes inhibited in the continuous presence of auxins[27].

Acclimatization of rooted plantlets was achieved by transferring them into plastic cups containing soil and farmyard manure using a mist chamber (Figure 1 *e*). Later, they were transferred to earthen pots containing soil and farmyard manure (Figure 1 *f*). The per cent survival decreased to 90 after three months and remained unchanged thereafter. The plant height increased significantly ($P \leq 0.05$) with time (Table 4). The average number of leaves, shoot diameter and number of nodes also increased with time, indicating successful establishment of tissue culture-raised plantlets (Table 4).

The present study is a report of *in vitro* propagation of *Q. floribunda* wherein cotyledonary nodes have been utilized as initial explants for multiple shoot formation, followed by rooting of individual microshoots. This method of micropropagation is proposed as a useful supplement to the conventional propagation methods used for the establishment of seedling nurseries. Since a large number of seeds have been used initially, it should also help to maintain, to some extent, germplasm diversity and not lead to genetic pauperization often attributed to tissue culture-raised 'clonal' plantations.

1. Chalupa, V., in *Somatic Embryogenesis in Woody Plants* (eds Jain, S., Gupta, P. and Newton, R.), Kluwer, Dordrecht, 1995, vol. 2, pp. 67–87.
2. Singh, J. S. and Singh, S. P., *Forest of Himalaya*, Gyanodaya Prakashan, Nainital, 1992.
3. Bhardwaj, D. R., Mishra, V. K. and Shamet, G. S., *J. Tree Sci.*, 1996, **15**, 49–51.
4. Tamta, S., Purohit, V. K., Nandi, S. K. and Palni, L. M. S., *Indian J. For.*, 2000, **23**, 135–138.
5. Bisht, M. S., Vyas, P., Bag, N. and Palni, L. M. S., in *Plant Tissue Culture and Molecular Biology – Applications and Prospects*, Narosa Publishing House, New Delhi, 1998, pp. 126–170.
6. Vieitez, A. M., San-Jose, M. C. and Vieitez, E., *J. Hortic. Sci.*, 1985, **60**, 99–106.
7. Favre, J. M. and Juncker, B., *Plant Cell Tissue Org. Cult.*, 1987, **8**, 49–60.
8. Chalupa, V., *Biol. Plant.*, 1988, **30**, 414–421.
9. San-Jose, M. C., Vieitez, A. M. and Ballester, A., *Silvac. Genet.*, 1990, **39**, 50–55.
10. Pardos, J. A., in Coll Int Culture *in vitro* des essences forestieres, (ed. AFOCEL), IUFRO, Fontainebleau, France, 1981, pp. 186–190.
11. Bellarosa, R., in *Biotechnology in Agriculture and Forestry* (ed. Bajaj, Y. P. S.), Springer-Verlag, Berlin, 1989, vol. 5, pp. 387–401.

12. Manzanera, J. A. and Pardos, J. A., *Plant Cell Tissue Org. Cult.*, 1990, **21**, 1–8.
13. Bennet, L. K. and Davies, F. T., *HortScience*, 1986, **21**, 1045–1047.
14. Sato, T., Mori, N. and Saito, A., *J. Jpn. For. Soc.*, 1987, **69**, 113–117.
15. Ide, Y. and Yamamoto, S., *ibid*, 1987, **69**, 109–112.
16. Schwarz, O. J. and Schlarbaum, S. E., *Ann. Sci. For.* (*50 Suppl.*), 1993, **1**, 340s–343s.
17. Purohit, V. K., Tamta, S., Chandra, S., Vyas, P., Palni, L. M. S. and Nandi, S. K., *Plant Cell Tissue Org. Cult.*, 2002, **69**, 121–133.
18. Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, Oxford and IBH, New Delhi, 1967.
19. Wilkinson, L., SYSTAT: The System for Statistics, 1L: Systat, Eranston, 1986.
20. Bressan, P. H., Kim, Y. J., Hyndman, S. E., Hasegawa, P. M. and Bressan, R. A., *J. Am. Soc. Hortic. Sci.*, 1982, **107**, 979–990.
21. Perinet, P. and Lalonde, K., *Plant Sci. Lett.*, 1983, **29**, 9–17.
22. Hutchinson, J. F., *Sci. Hortic.*, 1984, **22**, 347–358.
23. Chalupa, V., *Biol. Plant.*, 1984, **26**, 374–377.
24. Nandi, S. K., Palni, L. M. S., Letham, D. S. and Wong, O. C., *Plant Cell Environ.*, 1989, **12**, 273–283.
25. Letham, D. S. and Palni, L. M. S., *Annu. Rev. Plant Physiol.*, 1983, **34**, 163–197.
26. Bag, N., Chandra, S., Palni, L. M. S. and Nandi, S. K., *Plant Sci.*, 2000, **156**, 125–135.
27. Torrey, J. G., *Annu. Rev. Plant Physiol.*, 1976, **27**, 435–459.

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Effect of fire on nutrient dynamics in a semi-arid grazing land ecosystem of Madurai

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This study deals with the effect of fire on the nutrient dynamics of a grazing land in the semi-arid region of Madurai. The nutrient concentrations were maximum in the live shoot followed by below-ground parts, dead shoot and litter. The various nutrients accumulated in the plant components of the burned grazing land were in the order of N > K > Ca > Mg > P, whereas in the unburned site the nutrients were accumulated in the order of N > Ca > K > Mg > P. Except potassium, all the nutrients were higher in the unburned than in the burned grazing land. The uptake of nutrients from the soil was significantly higher ($P < 0.05$) in the unburned grazing land than in the burned grazing land. Of the total uptake, a larger amount of nutrients was transferred to above-ground parts than below-ground parts. In both the sites, the amount of nutrients returned to the soil through below-ground parts was more than through above-ground litter decomposition. The input to output nutrient ratio showed that burning of vegetation accelerates nutrient cycling.

GRASSLANDS are influenced by various biotic stresses such as grazing, mowing and fire. These stresses have tremendous impact in shaping or altering the vegetation. Fire alters the physical and chemical properties, organic and mineral reserves[1] and the microbial population of the soil[2]. The effects of fire on the nutrients of an ecosystem depend on the type and frequency of fire, the fuel load, time and season of burn, nature of the plant tissues burnt, topography, successional status of the community and post-fire climatic and biotic conditions acting thereupon[3,4]. A fire of mild intensity may stimulate high seedling establishment and growth by raising the soil temperature and nutrient status by removal of plant litter and vegetation cover[5]. As a result of fire, the availability of nutrients for plant uptake increases or they can be volatilized and lost from the site. Burning of vegetation has been used as a management practice to improve the productivity of an ecosystem. There are many reports available on forest fires[6,7]. However, reports on tropical grassland fires are scanty. The objective of the experiment was to study the effect of fire on nutrient dynamics of grazing land in the semi-arid region of Madurai.

The study was conducted at Madurai which is located at 10°00′N latitude and 78°10′E longitude. The climate of Madurai is semi-arid. The mean maximum temperature ranges from 28.2 to 37.4°C and the mean minimum temperature ranges from 20.5 to 28.3°C during the study period. Total annual rainfall during the study period was 1061 mm and the relative humidity ranged from 33 to 79%. The soil is a reddish-brown, laterite sandy loam of recent origin. The mean monthly pH of the soil ranged from 7.0 to 7.9 in the unburned site and 7.3 to 8.3 in the burned grazing land during the study period. *Heteropogon contortus* is the major dominant species of the grasslands. An area of about 0.2 ha of the grassland was intentionally burned on 25 May 1993. The adjacent unburned area of grassland of 0.25 ha was kept as control for the present study. The experiment was carried out for one year during July 1993 to June 1994.

The biomass was estimated by harvest method[8]. The vegetation was harvested at monthly intervals from ten randomly laid quadrats, each of 0.5 m^2. The plant

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