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Identifying suitable arable weeds as reference plants for measuring N_2 fixation in grain legumes in Zambia using ^{15}N natural abundance

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Abstract

Three options were evaluated for identifying suitable arable weeds as reference plants for estimating N_2 fixation in grain legume symbioses in 3 agro-ecological zones of Zambia using ^{15}N natural abundance. For each test legume species, 5 different small-farmer fields were randomly selected at each site, and 5 shoots of legume and 5 shoots of at least 3 wild weed species were randomly harvested from each of the 5 fields per site using a paired sampling protocol. The composition of weeds differed from site to site, and the types of weed species were found to increase with annual rainfall. Isotopic analysis of ^{15}N showed that 97% of the weed species were suitable as reference plants for estimating N_2 fixation at their sites of origin. No weed species was found with similar ^{15}N content across all sites. Within each site, some species were more dominant than others. The mean $\delta\ ^{15}N$ values for these dominant species ranged from –0.50 to 3.00‰, 4.31 to 5.56‰, and 5.39 to 6.64‰, respectively, for Kabwe, Kasama, and Magoye, with the differences between species means being significant ($P<0.05$) at only Kabwe. When the $\delta\ ^{15}N$ values were averaged across species, the means ranged from 1.06 to 2.66‰, 2.86 to 5.34‰, and 3.94 to 8.89‰, respectively for the Kabwe, Kasama and Magoye sites, with the differences being significant ($P\leq0.05$) at only Magoye. The combined mean $\delta\ ^{15}N$ value for herbaceous weeds was significantly greater and, with a lower standard error than that of graminaceous species at Kasama and Magoye, suggesting that, as reference plants, the herbaceous species were more likely to provide accurate estimates of N_2 fixation for these legume symbioses at the study sites. Selection of reference plants on a per field basis was the only option applicable to all sites, while selection based on suitable weed species across fields was an option for only Kabwe and Magoye, but not for Kasama. However, a $\delta\ ^{15}N$ value for the entire site, obtained from averaging the $\delta\ ^{15}N$ values of all field means of a site, was also as appropriate for estimating N_2 fixation at Kabwe and Kasama as the average $\delta\ ^{15}N$ value calculated from means of suitable species at Kasama and Magoye. Based on the mean $\delta\ ^{15}N$ values, both graminaceous and herbaceous weeds were found to be equally suitable as reference plants for estimating N_2 fixation at Magoye. However, herbaceous weeds were probably better reference plants for accurately estimating %Ndfa at Kabwe and Kasama.

Keywords: Grain legumes, options, arable weeds, reference plants, ^{15}N natural abundance, N_2 fixation, mean $\delta\ ^{15}N$ values

1. Introduction

The ^{15}N natural abundance technique has been widely used for assessing the dependence of field grown grain and pasture legumes on atmospheric N_2 fixation for their N nutrition (Kohl et al., 1980; Yoneyama et al., 1986; Sanford et al., 1995; Unkovich et al., 1994; Bolger et al., 1995). The method is based on a small but significant difference in the $^{15}N/^{14}N$ ratio between atmospheric N_2 and soil nitrogen (Domenach and Corman, 1984; Shearer and Kohl, 1988; Peoples et al., 1997a). Generally soil N is more ^{15}N-enriched than atmospheric N_2, which has 0.3663 atom % ^{15}N. Therefore, as N_2 is progressively assimilated from the air through symbiotic N_2 fixation, there is a gradual decline in the ^{15}N composition of total N in tissues of the N_2-fixing legume compared to non-fixing plants, which depend on ^{15}N-enriched soil N (Shearer and Kohl, 1986; Handley and Raven; 1992; Peoples et al., 1997a). Though small, the difference is easily measured by mass spectrometry (Yoneyama et al., 1984; Shearer and Kohl, 1988; Unkovich et al., 1994).

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The assumptions and requirements of the technique, including sampling strategies, analytical procedures and limitations for field application, have been discussed elsewhere (Kohl et al., 1980; Handley and Raven, 1992; Pate et al., 1994; Unkovich, 1996; Peoples et al., 1997b). Essentially, the natural abundance method can be applied wherever ^{15}N enrichment of soil mineral N is significantly and consistently different from that of atmospheric N_2. Natural ^{15}N has a positive $\delta\ ^{15}N$ value when a plant sample is more ^{15}N enriched than atmospheric N_2 and negative if it has less ^{15}N than N_2 (Domenach and Corman, 1984; Unkovich, 1996).

Non-N_2-fixing plants have higher $\delta\ ^{15}N$ values than N_2-fixing legumes, which obtain their N from both soil and the atmosphere (Domenach and Corman, 1984; Shearer and Kohl, 1986; Unkovich, 1996.) When using the ^{15}N natural abundance technique, a non-N_2-fixing reference plant is used to provide a measure of the ^{15}N enrichment of soil in which the legume is growing (Kohl et al., 1980, Peoples et al., 1997a). Reference plants also serve to integrate isotopic variations in soil ^{15}N composition occurring over time or with depth of soil from which the plant obtains its N (Shearer and Kohl, 1986; Pate et al., 1994; Peoples et al., 1997b). Thus, an appropriate reference plant should be similar to the legume in growth, phenology, rooting profile, and spatial or temporal pattern of soil N uptake and utilization (Peoples et al., 1997a). Although these attributes are difficult to quantify (Pate et al., 1994), naturally occurring weeds have been successfully used as reference plants for ^{15}N estimation of N_2 fixation under field conditions (Rerkasem et al., 1988; Sanford et al., 1995; Unkovich et al., 1994; Bolger et al., 1995; Maskey et al., 2001). Non-fixing isolines of legume species have also been used as suitable reference plants (Yoneyama et al., 1986). Pate et al. (1994) found that there was a change in ^{15}N value of legume shoots and weed species as the growing season progressed, suggesting that agroclimatic changes can affect the ^{15}N natural abundance methodology.

The precision of estimating N_2 fixation is enhanced when the $\delta\ ^{15}N$ values for legume and reference plant are different, and the $\delta\ ^{15}N$ value of the reference plant is at least 2‰ units greater than that of the legume (Pate et al., 1994; Peoples et al., 1997a). In fact, Unkovich et al. (1994) have shown that the precision associated with estimating N_2 fixation was progressively enhanced when ^{15}N enrichment of the non-N_2-fixing reference plant was increased while the ^{15}N enrichment of the legume approached zero. In addition, Unkovich (1996) also showed that the percent of plant nitrogen derived from the atmosphere (%Ndfa) could be estimated with ±10% accuracy when the reference plant ^{15}N enrichment was 10 times the analytical precision of measuring ^{15}N. Sampling legume and non-fixing plants in pairs further improves the accuracy of estimating N_2 fixation. This sampling protocol helps to maximize the similarity of the soil N pool from which the legume and non-fixing weed obtain their N (Shearer and Kohl, 1988; Peoples et al., 1997a). It is therefore recommended that the ^{15}N content of several potential reference plant species be analyzed (Shearer and Kohl, 1986; Unkovich, 1996) and the one closest to the required level of ^{15}N enrichment selected to match the legume. For each species, at least 5 individual plant samples should be obtained and analysed to account for real variation across the site.

This study investigates options for selecting reference plants, and identifies suitable weeds as reference plants for estimating N_2 fixation in grain legumes under low-input farming systems in Zambia.

2. Methods and Materials

Study sites

Field surveys were done during the cropping season of December 1998 to April 1999 in three geographically distinct sites, each representing an agroecological zone of Zambia (Veldcamp, 1984). Kasama, the first site, is located some 900 km north of Lusaka and within the high-rainfall zone (1,000 mm annually). Kabwe, the second site, is 160 km north of Lusaka in the medium-rainfall zone (800–1,000 mm per year). The third site, Magoye, is 160 km south of Lusaka in the low-rainfall zone (800 mm annually).

Field and plant selection

Small-farmer fields, predominantly monocropped and averaging 0.03 to 0.17 ha per household were used in this study. For each legume, plants were sampled from 5 replicate fields within 50 km radius of the farm block. Selection of the field for sampling was done by first numbering all the fields in the site and randomly selecting them by numbers. Each chosen farm was divided into roughly five equal sections. Plants were randomly sampled from each section by walking in a ziz-zag across the length of the field starting from one corner.

Plant harvest

A paired legume-reference plant sampling procedure was used similar to the method of Unkovich et al. (1994). In each section of the field, the shoot of a randomly selected legume was cut-off at the crown region and one plant from each of 3 to 5 weed species growing at about 30 cm away from the harvested legume, also sampled at the crown region. This way, 5 legume shoots and 5 shoots of each of the 3–5 common weed species were obtained from the field. Wherever possible, the same weed species were sampled at every site. The 5 shoots were pooled for the legume and for the weed species. The harvesting procedure was repeated at all selected fields in the 3 study sites. Volunteer maize and sorghum plants were also sampled and included as reference plants.

The pooled plant samples were oven-dried at 70°C, weighed, ground to a fine powder, thoroughly mixed, and stored in tightly capped plastic vials prior to ^{15}N analysis.

^{15}N analysis

About 2 mg of each sample was analyzed for ^{15}N content using a Finnigan MAT 252 mass spectrometer connected to a NAC 1500NC CHN analyzer. The tissue of a *Nasturtium* spp. was routinely included as internal N-containing standard for every 5 runs of plant samples. The $\delta\ ^{15}N$ values of internal standards were used to correct for machine error.

Statistical analysis

The error associated with ^{15}N measurement was estimated as the standard error of mean from the $\delta\ ^{15}N$ data of *Nasturtium*. The same data were also subjected to one-way analysis of variance (ANOVA) to detect possible differences among means for the 4 standard runs. The value of the standard error was compared with the $\delta\ ^{15}N$ values of weeds to assess their suitability as reference plants.

Statistical analysis was done for data from each site, using the plant sample from each field as a replicate. One-way ANOVA was performed using the statistical package of STATISTICA to detect differences between species and fields. A t-test was also done to compare the $\delta\ ^{15}N$ of graminaceous plants with herbaceous weeds.

3. Results

Precision of $\delta\ ^{15}N$ measurements

The mean $\delta\ ^{15}N$ values for *Nasturtium* were 6.94, 6.68, 7.80, and 6.86‰, respectively, for the 4 runs. The analysis of variance showed that the means were not significantly different. Consequently the combined standard error of the means (±0.06‰) was taken as the estimated error associated with sample analysis.

Weed species and their prevalence across all sites

In general the number and types of weed species increased from Magoye (low rainfall) to Kasama (high rainfall), thus following the annual rainfall pattern. In all, 10, 18, and 7 different weed species were sampled from Kabwe, Kasama, and Magoye, respectively (Fig. 1). At Kabwe, *Celosia trigyna* was the most common weed, occupying 89% of the fields; this was followed by *Zea mays* with 78%. At Magoye, 3 weed species were common, namely *Hibiscus meeusei* with 100% presence, *Bidens schimperi* 71%, and *Dactylocterium* sp., and *Z. mays* 57% each. All other weed species occurred in only 45% or less of the fields at each site. In contrast to Kabwe and Magoye, there were few weed species in Kasama, the most common being *Eleucine indica*, *Ceratotheca sesamoides*, and *Cleome hirta* which occurred in 20–33% of the fields.

Of all the 35 weed species sampled, only *B. schimperi*, *B. olitorius*, *Sorghum bicolor*, and *Z. mays* were found in all sites, but their $\delta\ ^{15}N$ values differed from site to site. At Kabwe the average $\delta\ ^{15}N$ values were 1.96, 1.98, 10.10, and –0.50‰; at Kasama 5.10, 7.13, 1.57, and 5.75‰; and at Magoye 6.64, 6.39, 5.63 and 5.85‰. *Closia trigyna* and *E. indica* were only found at Kabwe and Kasama, while *H. meeusei* occurred only at Kabwe and Magoye.

Table 1. Field mean $\delta\ ^{15}N$ values, statistical ranges and standard errors of weeds harvested at Kabwe, Kasama and Magoye. Each mean was averaged over weed species within the field.

Location	Field	Mean $\delta\ ^{15}N$ (‰)	Statistical range	S.E. (±‰)
Kabwe	C16	1.56 a (2.37)	3.45 (0.68)	0.82 (0.22)
	C25	1.34 a (2.31)	4.05 (0.31)	0.97 (0.09)
	C30	2.66 a (3.99)	4.97 (1.28)	0.67 (0.28)
	C34	1.46 a	2.67	0.66
	C36	1.09 a	2.51	0.44
Kasama	N03	2.86 a	2.30	0.67
	N05	5.75 a	2.18	0.69
	N07	5.00 a	2.46	0.55
	N08	5.34 a	3.16	1.03
	N09	5.28 a	0.16	0.05
	N11	5.06 a	1.15	0.33
Magoye	S01	7.04 a	1.90	0.36
	S02	6.04 a	1.03	0.14
	S04	5.63 b	0.65	0.14
	S08a	3.94 c	1.70	0.35
	S03	8.89 *	0.96	0.31
	S08b	5.48 *	5.38	1.57
	S09	8.60 *	10.60	3.11

Means with similar letters in a column within a site were not significantly different at P<0.05. Brackets are mean $\delta\ ^{15}N$ values when *Z. mays* was excluded; * Not statistically analyzed.

Table 2. Legume crops at the study sites, their mean $\delta\ ^{15}N$ values, statistical ranges, and standard errors of the means.

Location	Legume	Mean $\delta\ ^{15}N$ (‰)	Statistical range	S.E. (±‰)
Kabwe	*V. unguiculata*	–0.18	–0.23	0.50
	P. vulgaris	–0.19	4.91	0.85
	V. subterranea	–0.22	0.41	0.18
	A. hypogea	1.53	1.04	0.21
Kasama	*V. unguiculata*	0.54	1.94	0.32
	A. hypogea	1.59	5.34	0.99
	P. vulgaris	4.88	3.25	0.61
Magoye	*V. unguiculata*	1.16	3.33	0.55
	V. subterranea	2.20	2.05	0.49
	A. hypogea	3.39	3.38	0.67

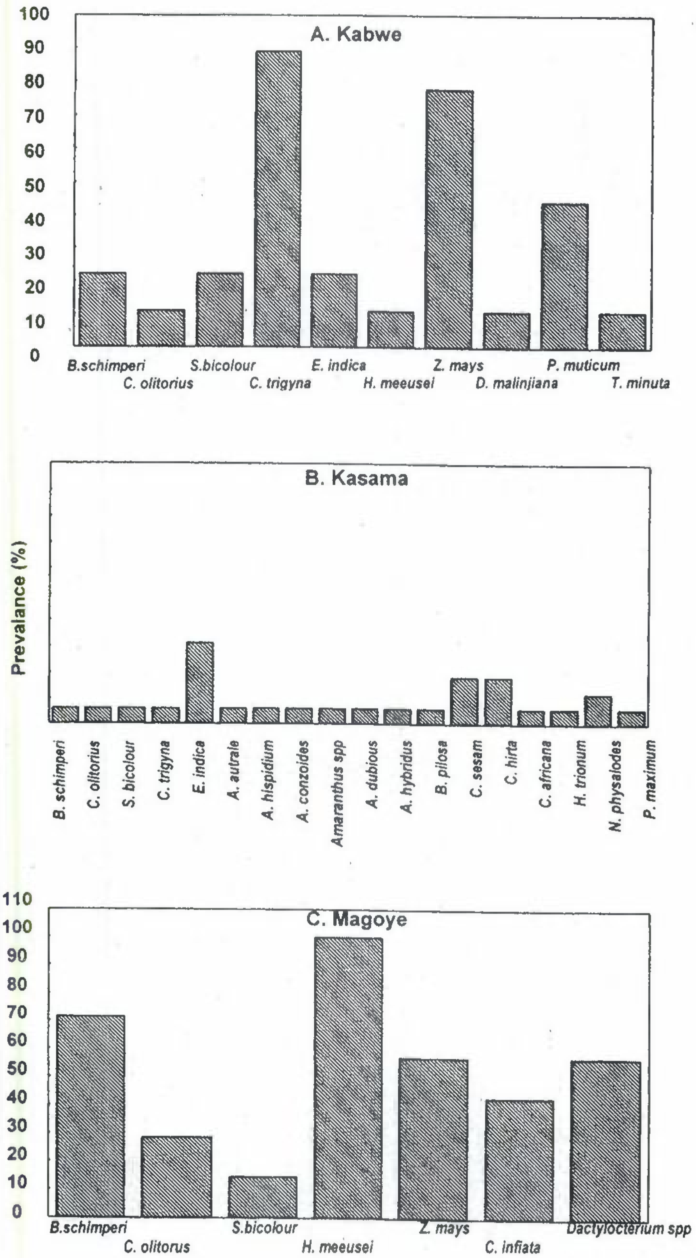

Figure 1. Prevalence of the various weed species in fields at the different study sites in a) Kabwe, b) Kasama, and c) Magoye.

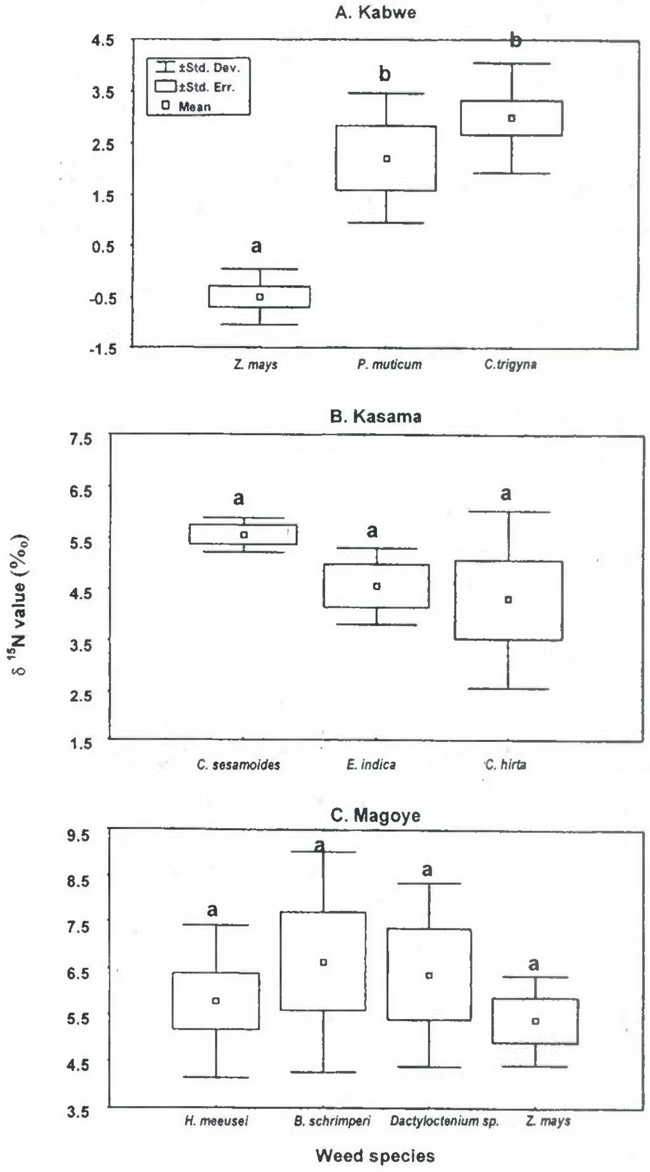

Figure 2. Mean δ ^{15}N values and standard errors for frequently encountered weed species in fields at a) Kabwe, b) Kasama, and c) Magoye. Bars with dissimilar letters are significantly different at P≤0.05.

Selections based on weed species across site

The data for the common weeds were grouped by species (Fig. 2). The mean δ ^{15}N values for all weed species, except maize (*Z. mays*) at Kabwe, were at least 10 times the value for the standard error of measurement (0.06‰). In most cases, the weeds were richer in ^{15}N by 2‰ units or more relative to legumes growing in the same field (data not shown), indicating that an accuracy of ±10% could be achieved if these weeds were used as reference plants for estimating %Ndfa. The very low negative δ ^{15}N values for *Z. mays* showed that it was not a suitable reference plant for measuring N_2 fixation at Kabwe.

The mean δ ^{15}N values at any one site did not differ between species (Fig. 2), except at Kabwe where the mean δ ^{15}N value of maize (*Z. mays*) was significantly lower than that of *C. trigyna* and *Panicum muticum* (Fig. 2A). Of the latter two species, *C. trigyna* had a larger δ ^{15}N value and lower standard error (S.E.) of the mean. The S.E. values suggest that *C. trigyna* would make a better reference plant than *P. muticum* for accurate estimation of N_2 fixation at Kabwe. In the same way, *C. sesamoides* with a larger mean

$\delta\ ^{15}N$ value and lowest standard error of the mean would be a better reference plant for estimating %Ndfa in the Kasama area than *C. hirta* and *E. indica* even though the 3 species had similar $\delta\ ^{15}N$ values (Fig. 2B). The data for Magoye (Fig. 2C) were similar to those for Kasama. While the standard error value favoured *Z. mays* as the most suitable reference plant for Magoye, actual estimates of %Ndfa based on all weeds would most likely be similar since their average ^{15}N enrichments were statistically the same (Fig. 2C).

Selections based on weed species within farm

Table 1 shows mean $\delta\ ^{15}N$ values and their statistical ranges and S.E. for some fields at each site. The mean $\delta\ ^{15}N$ values differed for fields at Magoye but not at Kabwe and Kasama. Due to variability in the composition of weed species and differences in numbers on each farm, sites could not be directly compared using statistical analysis. In general, however, weed ^{15}N enrichments differed considerably from site to site. The combined means of $\delta\ ^{15}N$ values for weed species were 1.51, 5.01, and 6.52‰, respectively, for Kabwe, Kasama and Magoye, while those for legumes were 0.19, 2.34, and 2.25‰, respectively for the 3 sites (Table 2). Due to the strong differences between sites as a result of variations in soil ^{15}N enrichment, it would be inappropriate to use reference plants from one site to assess N_2 fixation at another site.

Although it was not possible to statistically analyse for plant-to-plant variation within a species due to pooling of samples, within-field differences in ^{15}N composition of weed species were quite apparent as shown in Fig. 3 for 2 fields from each site. At Kabwe, for example, the $\delta\ ^{15}N$ values ranged from –0.85‰ for *Z. mays* to 2.59‰ for *P. maximum* in field C16. Differences were also observed between farms in the same site. The mean $\delta\ ^{15}N$ values for fields N03 and N05 at Kasama were 2.86 and 5.74‰, respectively, while at Magoye it was 4.79‰ for field S09, and 9.79‰ for field S03 (Fig. 3C). Thus, 2 weed species from the same site did not necessarily provide the same ^{15}N enrichment value for the soil.

Selection of an appropriate reference plant would depend on the $\delta\ ^{15}N$ value of each weed relative to that of the legume; the larger the difference between the 2, the higher the accuracy in estimating %Ndfa. However, an average $\delta\ ^{15}N$ value for all species within a field would probably more realistically reflect the ^{15}N composition of the soil. But this should only apply when the $\delta\ ^{15}N$ values of individual weeds are close (see field S09 Fig. 3). If there are large differences in isotopic composition between the potential reference plants (e.g. field C16, at Kabwe) then the $\delta\ ^{15}N$ values of individual species must be used.

Graminaceous versus herbaceous weeds

Data for graminaceous and herbaceous weeds were analysed separately and their means and standard errors presented in Figs. 4A–C. Though not significant at Magoye, the mean differences for the 2 types of weeds were significant at Kasama and Kabwe, where the average ^{15}N enrichments were greater for herbs than grasses. The latter had higher standard error of mean compared to the herbaceous species, indicating greater variability in ^{15}N enrichment among the grasses relative to the herbs. The data suggest that herbaceous species were more accurate in representing soil ^{15}N enrichment at Kasama and Kabwe though both graminaceous and herbaceous plants were equally suitable as reference material at Magoye. The grass species however had lower standard error of means at Magoye compared to the herbs. Maize ^{15}N enrichment undoubtedly contributed more than any other species to the lower $\delta\ ^{15}N$ values for graminaceous species at Kabwe (Fig. 1). When the ^{15}N values for maize were discarded, the average $\delta\ ^{15}N$ value for the field soil was considerably increased, and the standard errors for the same means reduced (Table 1).

4. Discussion

To effectively tap the agronomic and economic benefits of symbiotic legumes in traditional cropping systems in Africa requires reliable estimates of the amount of nitrogen fixed under field conditions (Vance and Graham, 1995; Dakora and Keya, 1997; Keya, 1998; Rerkasem et al., 1988; Maskey et al., 2001). For food grain legumes, the ^{15}N natural abundance technique has offered realistic measurements of N-fixed (Peoples et al., 1997a,b). However, certain conditions and requirements must be met in order to obtain meaningful results with this technique (Peoples et al., 1997a,b). In particular, the choice of appropriate reference plant to monitor soil ^{15}N levels is critical for estimating %Ndfa (Armstrong et al., 1994; Sandford et al., 1995; Rerkasem et al., 1988; Maskey et al., 2001). In this study, 3 possible options were assessed for selecting arable weeds as suitable reference plants for estimating N_2 fixation using ^{15}N natural abundance under field conditions in Zambia. Firstly the assessment of arable weeds for their suitability as reference plants and their ultimate selection were based on verifiable statistical criteria (Kohl et al., 1980; Domenach and Corman, 1984; Pate et al., 1994; Unkovich et al., 1994).

Secondly, quantifying the $\delta\ ^{15}N$ values of weed species showed that they were higher in magnitude than those of legumes, and thus indicated their suitability as reference plants for estimating N_2 fixation in legumes grown under small-farmer conditions in Zambia using the ^{15}N natural abundance technique. Additionally, the $\delta\ ^{15}N$ data also showed that the values obtained were site-dependent as a result of differences in soil ^{15}N composition between sites. So it was not possible to select one reference plant species that was suitable for all sites. However, a species-based

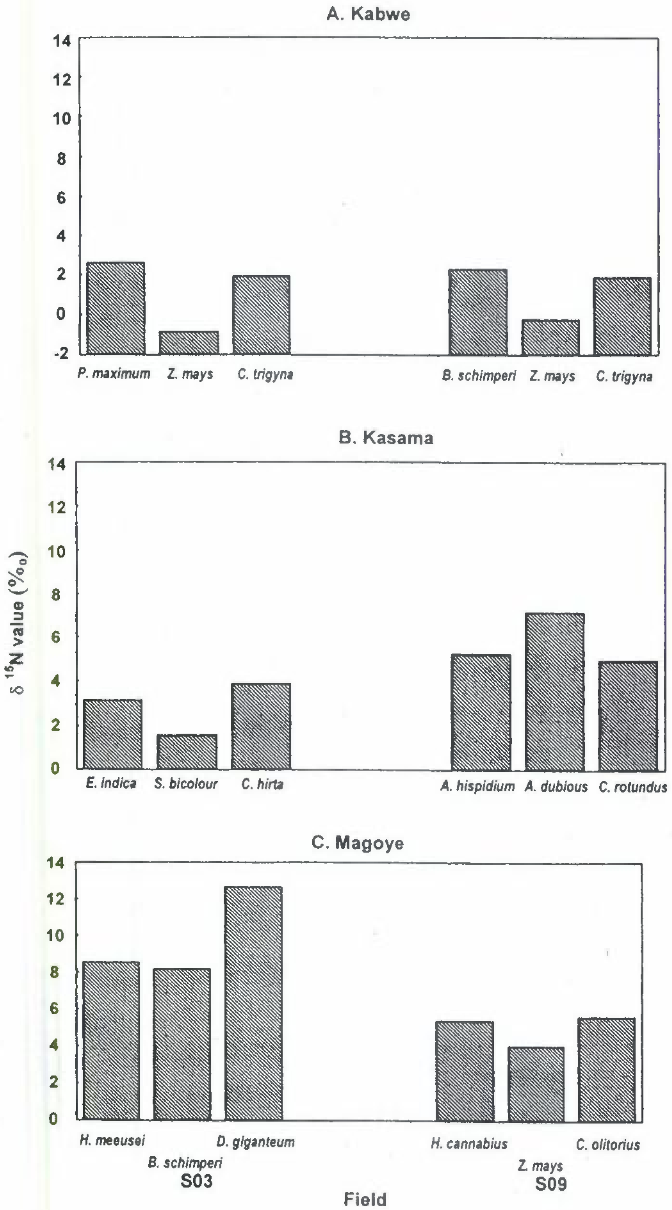

Figure 3. Variability of δ ^{15}N values for weed species in two selected fields at each study site in a) Kabwe, b) Kasama, and c) Magoye.

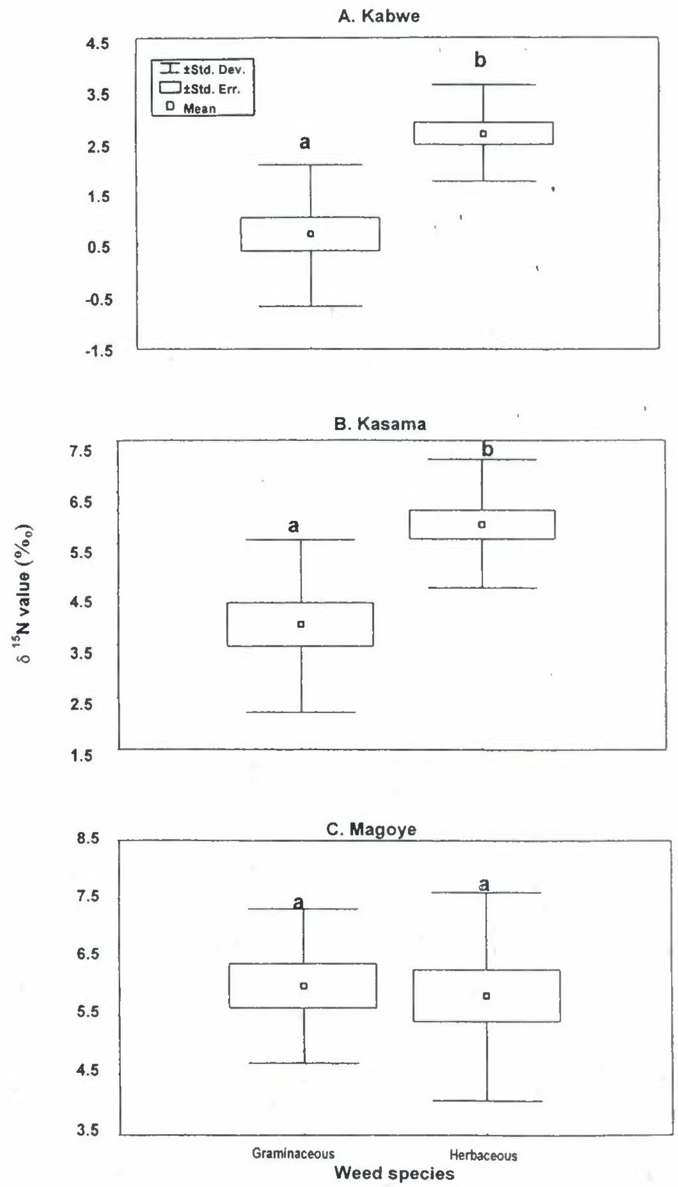

Figure 4. Mean δ ^{15}N values combined over species for graminaceous and herbaceous weed types for each study site at a) Kabwe, b) Kasama, and c) Magoye. Bars with dissimilar letters are significantly different at P≤0.05.

selection criterion was possible for Kabwe and Magoye, where *C. trigyna*, *B. schimperi* and *H. meeusei* were present in at least 70% of the fields at those sites (Table 1), and had relatively high δ ^{15}N values (Fig. 2). In contrast, the same was not possible at Kasama where the weed species composition was highly variable with none dominating in that area. An advantage with the species-based approach is that, it is less time-consuming and probably more cost-effective to use in large scale surveys.

A field-based approach was also assessed for its suitability in selecting reference plants. However, it was found to be time-consuming and resource-demanding. It requires assessing candidate reference plants from different species with differing growth and rooting habits, as well as with variable spatial and temporal utilization of available soil N. These differences were therefore, no doubt, the main cause of the variation in ^{15}N contents among weed species even from the same field. Despite these difficulties, the field-based strategy could be successfully used to select suitable reference plants at Kasama. Here, the combined means of δ ^{15}N values for all species provided a more realistic estimate of soil isotopic ^{15}N enrichment for the field than a value from a single species. At Kabwe, however, ^{15}N enrichments of the available weeds varied widely in some fields, thus rendering the mean value a less realistic estimate of soil ^{15}N (Fig. 3). Perhaps, in such instances, it would be better to measure the δ ^{15}N values of many species in the field, and then disregard the extreme values when calculating the field average for estimating N_2 fixation in the legume. The field-based approach therefore seems most suited for application to plants growing in heterogenous soils, where the soil ^{15}N contents easily vary between short distances within a site.

The results of this study also showed that the average ^{15}N composition of graminaceous weeds differed largely from that of herbaceous weeds, in that, the former had higher δ ^{15}N values relative to the latter, a finding consistent with earlier reports (Shearer and Kohl, 1988; Pate et al., 1994). This variation in the δ ^{15}N values of the 2 weed types probably stemmed from differences in their spatial and temporal utilization of soil nitrogen pools as shown for 2 of the 3 sites in this study. The differences in ^{15}N enrichment were however not discernable in Magoye (Fig. 4) and so either group, be they graminaceous or herbaceous, could therefore make an appropriate reference plant for estimating N_2 fixation in the grain legumes at that site. Interestingly, Bolger et al. (1995) observed a similar situation in one south-west Australian area in their search for suitable reference plants for estimating N_2 fixation. The data from this study (Fig. 4) suggest that at Kabwe and Magoye the herbaceous weeds were a better choice as reference plants, thus supporting the approach of first analysing available weeds for ^{15}N when selecting for suitable reference plants. Further, the choice of reference plant, whether graminaceous or herbaceous, must be determined for each site. Although there seemed to be no apparent relationship between rainfall intensity and soil δ ^{15}N as monitored by ^{15}N enrichments in weed and legume plants from different rainfall zones (Tables 1 and 2), the mean δ ^{15}N values were lower at Kabwe and highest at Magoye for both species. But whether this was due to differences in soil type or the prevalent farming practices, remains to be determined. Thus, although the average δ ^{15}N differed from site to site, it did not exhibit any pattern associated with seasonal rainfall of the sites where this study was conducted.

In conclusion, our data show that most weed species were suitable as reference plants at their sites of origin and that the appropriate option for selecting them for estimating N_2 fixation depended on the site. The very low δ ^{15}N values for *Z. mays* at Kabwe indicates that caution must be exercised when using this species as a reference plant for estimating N_2 fixation using δ ^{15}N natural abundance.

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