Genetic Assessment of *Amaranthus* Linn. Genotypes in Treatment Combinations of *Glomus clarum* and *Leucaena leucocephala* Lam. Using Simple Sequence Repeat (SSR) Marker

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**Abstract**

The molecular and genetic assessments of the seeds of five *Amaranthus* genotypes in four treatment combinations of Arbuscular mycorrhiza Fungus (*Glomus clarum*) and Green manure (*Leucaena leucocephala*) were evaluated using SSR marker with four primers. The experiment was laid out in a complete randomized design with four replicates. Heritability of growth traits were higher than yield traits for treated *Amaranthus* genotypes. The plant height had strong positive correlation with stem length (r=0.8919), inflorescent length (r=0.6150) and inflorescent width (r=0.6004). *Prin 1* accounted for the highest variation with mean value of 3.75. The highest allelic frequency of 0.75 was recorded for ASAAC005 and ASAAC006. However, ASAAC001 primer was polymorphic (66.86%), with highest allele number and allele diversity of 5.0000 and 0.7200 respectively. The dendrogram among the treated *Amaranthus* genotypes showed their phylogenetic relationship which can be useful in selection of closely related genotypes for desirable traits. Therefore, NGBO1234 and NGBO1271, *G. clarum* + *L. leucocephala* had the highest concentration of extracted DNA (4039.2ul), while NGBO1234 treated with *G. clarum* and NGBO1234 treated with *L. leucocephala* had highest genomic DNA nanodrop of 2.14.

**Keywords** *Amaranthus*; *Glomus clarum*; *Leucaena leucocephala*; SSR markers

**Background**

*Amaranthus* is a green vegetable that belongs to the family Amaranthaceae (Tucker, 1986; Bressani et al., 1992). It survives under harsh conditions, and is of economic, nutritional and medicinal values (Breene, 1991; Gupta and Gudu, 1991; Asfaw, 1997). Thus, the improvement of *Amaranthus* may be useful for alleviating hunger in developing nations, especially in overpopulated and undernourished areas (Pal and Khoshoo, 1974; Sauer, 1993).

The vegetable helps to build immunity in human, and ensure proper functionality of organs and tissues through provision of vitamins, minerals like calcium, iron and phosphorous and other phytochemicals (George, 2003; Nnamani et al., 2007). The fiber content also prevents constipation but consumed as a staple food during ancient times (Noonan, 1999; Buragohain et al., 2013). *Amaranthus* species such as *A. blitus*, *A. cruentus*, *A. hypochondriacus* are often planted for leaves, whereas *A. caudatus*, *A. hypochondriacus*, *A. cruentus*, *A. hybridus* are planted for their grain (Caselato-Sousa and Amaya-Farfan, 2012).

*Amaranthus* species consist of grain and weedy types, with the common grain types being *A. hypochondriacus*, *A. cruentus*, *A. caudatus* and the major weedy types include; *A. viridis*, *A. spinosus*, *A. retroflexus* and *A. hybridus*.

Arbuscular Mycorrhiza Fungi (AMF) plays an important role in the uptake of water, nutrients and improves crop performance and soil quality (Koske and Polson, 1984; Osonubi et al., 1995; Neveen et al., 2008). They help plants to capture nutrients such as phosphorus, sulphur, nitrogen and micronutrients from the soil (Brundrette,
Glomus clarum is one of the Arbuscular Mycorrhiza Fungi (AMF) that supports the growth and improves yields of plants (Olawuyi et al., 2012; Fapohunda et al., 2013).

Simple Sequence Repeat (SSR) is PCR-based marker with the highest information content when evaluating and characterizing the germplasm (Powell et al., 1996). They are reliable, reproducible, cheap and discriminative compared to other markers (Smith et al., 1997). Simple Sequence Repeat (SSR) works on the principle of microsatellite assay. It anchors based on the fact that, it is highly polymorphic even between closely related lines. It requires low amount of DNA and can be easily automated for high throughput screening which can be exchanged between laboratories but are highly transferable between populations (Gupta et al., 1999). SSRs are mostly co-dominant markers for studies on population genetics and mapping of genes (Jari and Lagoda, 1996; Goldstein and Schlotterer, 1999). The use of fluorescent primers in combination with automatic capillary or gel-based DNA sequencers had been adopted in most advanced laboratories, while SSR are excellent markers for fluorescent techniques, multiplexing and high throughput analysis.

Despite the global food insecurity, there is need to provide information on suitable SSR primer and bio inoculant to establish genomic relationship among Amaranthus genotypes. This will enhance the genetic management and conservation of Amaranthus.

This study investigated the molecular and phylogenetic relationship among Amaranthus germplasm in treatment combinations of Arbuscular Mycorrhiza Fungi (AMF) and Leucaena leucocephala (GM) using Simple Sequence Repeat (SSR) marker.

1 Results
The nanodrop and DNA concentration of the extracted Amaranthus genotypes were found to be at 260/280/gl in Table 1. The gel results showing the PCR Amplification of DNA in Amaranthus genotypes is presented in Figure 1. The Polyaclrylde Gel Electrophoresis (PAGE) of treated Amaranthus genotypes using primers; ASAAC001, ASAAC005, ASAAC006 and ASAAC011 are shown in Figure 2, Figure 3, Figure 4 and Figure 5 respectively.
Table 1 Nanodrop and DNA concentration of *Amaranthus* genotypes in treatment combinations of AMF (*Glomus clarum*) and GM (*Leucaena leucocephala*)

| S/N | Genotype/Treatment | Total Concentration of Genomic DNA Extracted(ul) | Nanodrop of Genomic DNA (260/280gl) |
|-----|--------------------|--------------------------------------------------|-------------------------------------|
| 1   | G1T1               | 1979.50                                          | 1.96                                |
| 2   | G1T2               | 1819.20                                          | 2.08                                |
| 3   | G1T3               | 1033.70                                          | 1.95                                |
| 4   | G1T4               | 4039.20                                          | 1.82                                |
| 5   | G2T1               | 2176.60                                          | 1.99                                |
| 6   | G2T2               | 1446.70                                          | 1.95                                |
| 7   | G2T3               | 788.80                                           | 2                                   |
| 8   | G2T4               | 991.80                                           | 1.88                                |
| 9   | G3T1               | 1846.60                                          | 1.78                                |
| 10  | G3T2               | 1436.60                                          | 2.14                                |
| 11  | G3T3               | 2766.80                                          | 1.92                                |
| 12  | G3T4               | 961.30                                           | 1.74                                |
| 13  | G4T1               | 1625.40                                          | 1.71                                |
| 14  | G4T2               | 1939.60                                          | 1.56                                |
| 15  | G4T3               | 2039.60                                          | 1.87                                |
| 16  | G4T4               | 2003.20                                          | 1.89                                |
| 17  | G5T1               | 2094.80                                          | 1.92                                |
| 18  | G5T2               | 2270.30                                          | 2.17                                |
| 19  | G5T3               | 1168.10                                          | 2.14                                |
| 20  | G5T4               | 1498.80                                          | 2.03                                |

Note: G1 = NGB01271; G2 = NGB01644; G3 = NGB01234; G4 = NGB01613; G5 = NGB01662; T1 = Control; T2 = AMF (*Glomus clarum*) only; T3 = GM only (*Leucaena leucocephala*); T4 = AMF + GM (*Glomus clarum* + *Leucaena leucocephala*)

The growth and yield of *Amaranthus* treated with GM (*Leucaena leucocephala*) are shown in Figure 6 and Figure 7. The quality of genomic DNA concentration was generally good with the highest total concentration of genomic DNA of 4039.20 ul recorded for combinations of *Glomus clarum* and *Leucaena leucocephala* on NGB01271. The Nanodrop of genomic DNA of *G. clarum* for genotype NGB01662 had the highest value of 2.17 gl followed by NGB01234 for *G. clarum* (2.14 gl) and NGB01662 for *L. leucocephala* (2.14 gl).

A total of four primers of SSR marker revealed the diversity and major allele frequency as well as the polymorphic information content of five *Amaranthus* genotypes in treatment combinations of *G. clarum* and *L. leucocephala* (Table 2). The result showed that primer ASAAC001 was highly polymorphic (66.86%) with the highest allele number and allele diversity of 5 and 0.7200 respectively compared with other primers. ASAAC005 and ASAAC006 primers had the highest major allele frequency of 0.7500. Four (4) amplified microsatellite loci were revealed by ASAAC001 and ASAAC005 primers while three (3) were produced by ASAAC006 and ASAAC011 primers.
Figure 6 Growth of *Amaranthus* cultivars treated with GM

Figure 7 The yield of *Amaranthus* cultivars treated with GM

Table 2 Frequency and diversity of allele and polymorphic information content (PIC) of treated *Amaranthus* Genotypes using SSR marker

| Marker     | Major Allele Frequency | Sample Size | Number of amplified microsatellite loci | Allele No | Allele Diversity | Percentage Polymorphic Information Content (%) |
|------------|------------------------|-------------|----------------------------------------|-----------|------------------|-----------------------------------------------|
| ASAAC001   | 0.3500                 | 20.0000     | 4.0000                                 | 5.0000    | 0.7200           | 66.86                                         |
| ASAAC005   | 0.7500                 | 20.0000     | 4.0000                                 | 4.0000    | 0.4150           | 38.94                                         |
| ASAAC006   | 0.7500                 | 20.0000     | 3.0000                                 | 3.0000    | 0.3950           | 34.70                                         |
| ASAAC011   | 0.6000                 | 20.0000     | 3.0000                                 | 3.0000    | 0.5600           | 49.92                                         |
| Total      |                        | 80.0000     | 14.0000                                | 15.0000   |                  |                                               |
| Mean       | 0.6125                 | 20.0000     | 3.5000                                 | 3.7500    | 0.5225           | 47.61                                         |

The result of Nei’s Coefficient of gene variation (GST), gene diversity per locus and total gene diversity are shown in Table 3. The mean total for Nei’s Coefficient of gene variation (GST), gene diversity per locus and total gene diversity were 0.2275, 0.3391, and 0.4389 respectively.

Table 3 Nei’s analysis of gene variation and gene diversity per locus in *Amaranthus* population

| Locus | Sample Size | Ht   | Hs   | Gst   |
|-------|-------------|------|------|-------|
| ID    | 80          | 0.4200 | 0.3000 | 0.2857 |
| =     | 80          | 0.4550 | 0.3750 | 0.1758 |
| 1     | 80          | 0.4997** | 0.4437** | 0.1119 |
| 0     | 80          | 0.3809** | 0.2375** | 0.3765 |
| Mean  | 80          | 0.4389 | 0.3391 | 0.2275 |

Note: Ht: Total gene diversity; Hs: Gene diversity per locus; Gst: Nei’s coefficient of gene variation or measure of genetic differentiation
The dendogram showing the phylogenetic relationships of *Amaranthus* genotypes in treatment combinations of *G. clarum* and *L. leucocephala* is shown in Figure 1. There are two major clusters sub-divided into six (6) groups in which clusters 4 and 6 had the highest number of genotypic treatment while cluster 1 had least with only NGBO1662 of *G. clarum* and *L. leucocephala* combinations. *Glomus clarum* treated with NGBO1271 is not closely related to untreated NGBO1644, NGBO1271 treated with *G. clarum* and *L. leucocephala*, untreated NGBO1271 and *L. leucocephala* treated with NGBO1271 in sub-cluster 6. G3T3, G3T1 and G3T2 are genetically related than G4T1 and G3T4 in cluster 4. G5T4 and G5T3 are closely related than G5T1 and G4T2, G5T2 in cluster 3 and G4T3 are genetically similar in cluster 2. Again, G2T2 and G2T3 are related compared to G2T4 (Figure 8)

Figure 8 Dendrogram showing the genetic relationships of the genotypes of treated *Amaranthus* spp

Note: G1 = NGBO1271; G2 = NGBO1644; G3 = NGBO1234; G4 = NGBO1613; G5 = NGBO1662; T1 = Control; T2 = AMF only; T3 = GM only; *(Leucaena leucocephala)*; T4 = AMF + GM
The result of the mean performance for genotypic effect of growth related characters for treated *Amaranthus* reveals significant (P<0.01) effect on *Amaranthus* genotypes as shown in Table 4. NGB01662 was significantly higher for plant height compared to other genotypes. While, number of leaves, stem length, stem girth, leaf length and leaf width were significantly influenced in NGB01271. Also, the plant height of NGB01644, and NGB01613 as well as NGB01234, NGB01644, NGB01613 and NGB01662 for number of leaves were not significantly different from one another.

Table 4 Genotypic effect on growth performance of *Amaranthus* in treatments combinations of *Glomus clarum* (AMF) and *Leucaena leucocephala* (GM)

| Genotype      | Plant height (cm) | Number of leaves | Stem length (cm) | Stem girth (cm) | Leaf length (cm) | Leaf width (cm) |
|---------------|-------------------|------------------|------------------|-----------------|------------------|-----------------|
| NGB01271     | 82.46^a           | 72.33^a          | 69.83^a          | 1.13^a          | 12.93^a          | 5.75^a          |
| NGB01644     | 77.54^b           | 51.08^ab         | 64.67^a          | 0.92^ab         | 11.75^ab         | 4.70^b          |
| NGB01234     | 82.29^ab          | 52.25^ab         | 68.88^a          | 0.91^ab         | 11.38^ab         | 5.08^ab         |
| NGB01613     | 77.25^b           | 57.33^ab         | 67.17^a          | 1.10^b          | 11.54^ab         | 4.95^b          |
| NGB01662     | 91.29^a           | 31.33^b          | 66.75^a          | 0.83^b          | 10.15^b          | 5.32^ab         |

Note: Mean with the same letter in the same column is not significantly at P≥0.05 according to Duncan Multiple Range Test (DMRT).

The result of the mean performance for genotypic effect of yield related characters for *Amaranthus* reveals significant (P<0.01) effect on *Amaranthus* genotypes (Table 5). NGB01662 was significantly higher for inflorescence length and inflorescence width compared to other genotypes. Also, the numbers of inflorescence were significantly higher for NGB01234 but different from other genotypes. NGB01271 is significantly higher for wet yield of inflorescent, dried yield of inflorescent, wet leaf and dried leaf than other genotype while number of branches was significantly higher in NGB01613.

Table 5 Genotypic effect on yield characters of *Amaranthus* treated with *Glomus clarum* and *Leucaena leucocephala*

| Genotype      | Inflorescence length (cm) | Inflorescent width (cm) | Number of inflorescence | Number of branches | Wet yield of inflorescent (g) | Dried yield of inflorescent (g) | Wet leaf (g) | Dried leaf (g) |
|---------------|---------------------------|-------------------------|--------------------------|-------------------|-------------------------------|----------------------------------|--------------|---------------|
| NGB01271     | 18.25^b                   | 3.96^b                  | 24.67^b                  | 11.00^ab          | 7.20^a                        | 2.25^a                           | 9.50^a       | 2.23^a        |
| NGB01644     | 22.08^b                   | 4.08^b                  | 31.42^ab                 | 9.92^b            | 4.64^b                        | 0.35^b                           | 5.39^b       | 1.41^b        |
| NGB01234     | 20.88^b                   | 4.00^ab                 | 33.17^a                  | 9.00^b            | 3.08^b                        | 0.23^b                           | 6.86^ab      | 1.56^ab       |
| NGB01613     | 15.29^c                   | 3.54^b                  | 28.92^b                  | 12.08^a           | 2.18^c                        | 0.34^b                           | 8.25^ab      | 1.79^ab       |
| NGB01662     | 29.83^c                   | 5.33^c                  | 31.08^ab                 | 2.42^c            | 3.79^b                        | 0.39^b                           | 5.99^b       | 1.45^b        |

Note: Means with the same letter in the same column are not significantly at P≥0.05 according to Duncan Multiple Range Test (DMRT).

The plant height, stem length, and leaf width was significantly (p<0.05) increased for *Leucaena leucocephala* (Green Manure) than other treatments and control (Table 6).

Table 6 Effect of AMF, GM and combinations of AMF + GM on growth characters of *Amaranthus*

| Treatment     | Plant height (cm) | Number of leaves | Stem length (cm) | Stem girth (cm) | Leaf length (cm) | Leaf width (cm) |
|---------------|-------------------|------------------|------------------|-----------------|------------------|-----------------|
| Control      | 78.73^bc          | 60.93^c          | 66.97^ab         | 1.04^a          | 12.42^a          | 5.19^ab         |
| AMF          | 78.57^bc          | 46.40^b          | 62.40^ab         | 0.87^b          | 10.74^b          | 4.59^ab         |
| GM           | 90.53^a           | 58.80^a          | 73.20^a          | 1.03^a          | 11.87^ab         | 5.53^a          |
| AMF + GM     | 80.83^b           | 45.33^b          | 67.27^ab         | 0.97^ab         | 11.17^ab         | 5.33^ab         |

Note: Mean with the same letter in the same column are not significantly at P≥0.05 according to Duncan Multiple Range Test (DMRT); AMF = *Glomus clarum*; GM = *Leucaena leucocephala*

The result in Table 7 shows that *Glomus clarum*, Arbuscular Mycorrhiza Fungus (AMF) is significantly higher for Inflorescence length while *Leucaena leucocephala* (Green Manure) is significantly higher for Inflorescence width, wet yield of inflorescent, dried yield of inflorescent and dried leaf. Also, the combined treatments of AMF + GM is significantly higher for number of inflorescence and wet leaf, while untreated plant was higher for number of branches.
The correlation result in Table 10 showed that plant height was positive and strongly correlated with stem length (r= 0.89, p≤0.01), inflorescent length (r= 0.62), inflorescent width (r= 0.60) and a positive correlation with leaf width (r= 0.54). Number of leaf had a strong significant positive correlation with stem girth (r= 0.68), leaf length (r= 0.61), number of branches (r= 0.77), wet leaf (r= 0.61) and a positive correlation with dried leaf (r= 0.57), stem length had a positive significant correlation with stem girth (r= 0.64), leaf width (r= 0.65) and dried leaf (r= 0.60) but a positive correlation with leaf length (r= 0.52) and wet leaf (r= 0.59). Stem girth is positive and strongly associated with leaf length (r= 0.75), leaf width (r= 0.64), number of branches (r= 0.69), wet leaf (r= 0.77) and dried leaf (r= 0.73). Leaf length is also positive and related with leaf breadth (r= 0.67) and wet leaf (r= 0.60) but a positive correlation with number of branches (r= 0.58), and dried leaf (r= 0.59). Leaf width had a significant positive correlation with wet leaf (r= 0.57) and dried leaf (0.55). Inflorescent length had a strong significant positive correlation with inflorescent width (r= 0.91) and number of inflorescent (r= 0.73). Inflorescent width had a strong significant positive correlation with number of inflorescent (r= 0.78). Wet yield of inflorescent had a

| Source of variation     | Genotypic variance (σ²g) | Phenotypic variance (σ²p) | Heritability |
|-------------------------|--------------------------|-------------------------|-------------|
| Plant height            | -3.607                   | 394.006                 | 0.009       |
| Number of leaf          | 619.267                  | 1359.907                | 0.455       |
| Stem length             | -74.869                  | 197.822                 | -0.378      |
| Stem girth              | 0.054                    | 0.089                   | 0.610       |
| Leaf length             | 2.402                    | 6.959                   | 0.345       |
| Leaf width              | 0.241                    | 1.421                   | 0.169       |
| Number of inflorescent  | -102.558                 | 333.257                 | 0.306       |
| Inflorescent length     | 49.006                   | 259.016                 | 0.189       |
| Inflorescent width      | -0.124                   | 5.734                   | -0.022      |
| Number of branches      | 48.675                   | 75.508                  | 0.645       |
| Wet leaf                | 6.886                    | 19.954                  | 0.345       |
| Dried leaf              | 0.233                    | 0.902                   | 0.259       |
| Wet yield of inflorescent | 11.368                 | 21.434                  | 0.530       |
| Dried yield of inflorescent | 2.642                 | 3.576                   | 0.739       |
strong significant positive correlation with dried yield of inflorescent (r= 0.69).

Table 9 Principal components analysis (PCA) of growth and yield characters of treated Amaranthus genotypes

| Characters       | Prin. 1 | Prin. 2 | Prin. 3 | Prin. 4 | Prin. 5 | Prin. 6 | Prin. 7 | Prin. 8 | Prin. 9 | Prin. 10 | Prin. 11 | Prin. 12 | Prin. 13 | Prin. 14 |
|------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|----------|----------|----------|----------|----------|
| PH               | 0.3213  | 0.2448  | -0.455  | -2.405  | 0.1096  | -0.4250 | 0.0614  | 0.0845  | 0.1129  | -0.0738  | -0.0771  | 0.0112   | 0.0038   | -0.7409  |
| NL               | 0.2932  | 0.1679  | 0.1027  | 0.4873  | -0.329  | -0.1187 | -0.3491 | 0.6587  | -0.0249 | 0.0652   | -0.2049  | -1.0205  | -0.0661  | 0.0353   |
| SL               | 0.3477  | 0.0373  | -0.373  | 0.2819  | -0.548  | -0.5079 | 0.2029  | -0.0475 | 0.1689  | 0.0177   | -1.0201  | -0.2275  | 0.0180   | 0.6093   |
| SG               | 0.3498  | -0.1925 | 0.0097  | 0.0149  | 0.0944  | 0.1018  | 0.2977  | -0.1568 | -0.7461 | -0.0416  | -0.3384  | 0.1098   | -0.1403  | -0.0563  |
| LL               | 0.3298  | -0.0853 | 0.1019  | -0.0463 | 0.5291  | 0.2546  | -0.0387 | 0.0202  | 0.3129  | -0.6449  | -0.0189  | 0.0994   | 0.0031   | 0.0262   |
| LB               | 0.3096  | -0.0586 | 0.1563  | -0.4388 | 0.3999  | 0.0822  | -0.3139 | 0.0994  | -0.1249 | -0.4952  | 0.3781   | 0.0690   | 0.0259   | 0.0195   |
| IL               | 0.0972  | 0.5065  | -0.1653 | 0.1177  | -0.1111 | -0.1298 | -0.1463 | 0.0736  | -0.1449 | -0.0837  | 0.1311   | 0.7236   | -0.685   | 0.2518   |
| IB               | 0.1439  | 0.4851  | -1.457  | 0.0704  | -0.701  | 0.1599  | -0.0014 | 0.0952  | -0.3491 | -0.2285  | 0.3857   | -0.5659  | 0.1771   | 0.0599   |
| NI               | 0.1200  | 0.3819  | -0.3850 | 0.2273  | 0.2777  | 0.2853  | 0.1929  | -0.0622 | 0.2492  | 0.4976   | -0.3436  | -0.0886  | -0.0501  | -0.0452  |
| NB               | 0.2511  | -0.2399 | -0.0521 | 0.5608  | 0.1223  | -0.3025 | 0.2288  | -0.3381 | 0.0872  | 0.1169   | 0.5052   | 0.0454   | 0.0811   | -0.0632  |
| Wet yield of inflorescent | 0.1295  | 0.2942  | 0.5510  | 0.1919  | 0.0319  | -0.0139 | -0.4369 | -0.5373 | 0.0379  | -0.359   | -0.2419  | -0.1143  | -0.0325  | -0.0043  |
| Dried yield of inflorescent | 0.0481  | 0.2254  | 0.6665  | 0.0415  | 0.0244  | 0.1738  | 0.5838  | 0.2950  | 0.1377  | 0.0311   | 0.0989   | 0.1079   | 0.0054   | 0.0289   |
| Wet leaf         | 0.3449  | -0.1455 | 0.0243  | -0.389  | -0.4317 | 0.3272  | -0.0371 | -0.0709 | 0.1162  | 0.0649   | -0.0928  | 0.1607   | 0.7111   | 0.0077   |
| Dried leaf       | 0.3423  | -0.0942 | -0.557  | 0.0723  | -0.4876 | 0.3354  | -0.0206 | -0.9975 | 0.2034  | 0.0149   | 0.1948   | -0.0499  | -0.6504  | 0.0163   |
| Eigenvalue       | 5.8765  | 3.2407  | 1.4199  | 0.9220  | 0.7089  | 0.6799  | 0.4201  | 0.2363  | 0.1654  | 0.1454   | 0.0934   | 0.0465   | 0.0364   | 0.0086   |
| Proportion       | 0.4197  | 0.2315  | 0.1014  | 0.0659  | 0.0506  | 0.0486  | 0.0300  | 0.0169  | 0.0118  | 0.0014   | 0.0067   | 0.0033   | 0.0026   | 0.0006   |

Note: PH: Plant height; NL: Number of leaves; SL: Stem length; SG: Stem girth; LL: Leaf length; LB: Leaf width; NI: Number of inflorescent; IL: Inflorescent length; IB: Inflorescent width; NB: Number of branches

2 Discussions

Genetic assessment and improvements in Amaranthus germplasm in treatment combinations will pay a key role in future studies and improvements of vegetable crop.

G1T4 had highest concentration of extracted DNA while G3T2 had highest genomic DNA nanodrop. This implies that good quality extracted DNA is enhanced when Amaranthus is treated with combinations of AMF and GM.

When treated singly with Glomus clarum and Leucaena leucocephala and a combination of AMF and GM, NGB01662 produced high mean for Inflorescence length and inflorescence width compared to other genotypes. Also, NGB01234 produced high mean for Number of inflorescence compared to other genotypes. NGB01271 produced high mean for wet yield of inflorescent, dried yield of inflorescent, wet leaf and dried leaf than other genotype while NGB01613 produced high mean for number of branches. Moreover, the treatments had a very high mean for the number of inflorescence, inflorescence length, inflorescence breadth, number of branches, fresh leaf biomass, and dried leaf biomass, wet yield of inflorescent and dried yield of inflorescent.

Also, NGB01662, NGB01644 and NGB01234 produced a higher mean for plant height and stem length compared to other genotypes. plant height, number of leaves, stem length, stem girth, leaf length and leaf breadth produced high mean for NGB01271 while NGB01613 produced high mean for plant height, number of leaves, stem length and stem girth. However, NGB01271 performed best for growth and yield in treatment combinations of AMF and GM. This supported the findings made by Olawuyi et al., (2012) and Babajide et al., (2012).

AMF produced high mean for inflorescence length. GM produced high mean for Inflorescence width, wet yield of inflorescent, dried yield of inflorescent and dried leaf. AMF + GM produced high mean for number of inflorescence and wet leaf while CONTROL produced high mean for number of branches. The performance of GM implies that it can be harnessed for agricultural productivity in order to reduce the effect of chemical fertilizers as reported by Sobulo (2000).
Table 10 Correlation co-efficient among characters in treatments of AMF and GM in *Amaranthus* genotype

|          | PH      | NL       | SL        | SG        | LL       | LB       | IL       | IB       | NI       | NB       | Wet yield of inflorescent | Dried yield of inflorescent | Wet leaf | Dried leaf | Genotype | Treatment |
|----------|---------|----------|-----------|-----------|----------|----------|----------|----------|----------|----------|---------------------------|-------------------------------|----------|------------|----------|-----------|
| PH       | 0.26    |          |           |           |          |          |          |          |          |          |                           |                               |          |            |          |           |
| NL       | 0.89**  | 0.42     |           |           |          |          |          |          |          |          |                           |                               |          |            |          |           |
| SL       | 0.40    | 0.68**   | 0.64**    |           |          |          |          |          |          |          |                           |                               |          |            |          |           |
| SG       | 0.37    | 0.61**   | 0.52*     | 0.75**    |          |          |          |          |          |          |                           |                               |          |            |          |           |
| LL       | 0.54*   | 0.40     | 0.65**    | 0.64**    | 0.67**   |          |          |          |          |          |                           |                               |          |            |          |           |
| LB       | 0.62**  | -0.11    | 0.24      | -0.18     | -0.05    | -0.02    | 0.10     | 0.10     | 0.91**   |          |                           |                               |          |            |          |           |
| IL       | 0.60**  | -0.07    | 0.25      | -0.02     | 0.10     | 0.10     | 0.10     | 0.10     | 0.91**   |          |                           |                               |          |            |          |           |
| IB       | 0.41    | -0.05    | 0.16      | 0.01      | 0.24     | 0.05     | 0.05     | 0.05     | 0.73**   | 0.78**   |                           |                               |          |            |          |           |
| NI       | 0.16    | 0.77**   | 0.42      | 0.69**    | 0.58*    | 0.25     | -0.23    | -0.20    | -0.03    |          |                           |                               |          |            |          |           |
| NB       | 0.31    | 0.17     | 0.13      | 0.13      | 0.15     | 0.11     | 0.19     | 0.01     | 0.04     | 0.69**   |                           |                               |          |            |          |           |
| Wet yield of inflorescent | 0.11 | 0.17 | 0.07 | 0.13 | 0.13 | 0.15 | 0.11 | 0.19 | 0.01 | 0.04 | 0.69** |
| Dried yield of Inflorescent | 0.46 | 0.61** | 0.59* | 0.77** | 0.60** | 0.57* | -0.05 | 0.09 | 0.05 | 0.49 | 0.15 | 0.06 |
| Wet leaf | 0.49 | 0.57* | 0.60** | 0.73** | 0.59* | 0.55* | 0.03 | 0.17 | 0.10 | 0.46 | 0.18 | 0.08 | 0.95 |
| Dried leaf | 0.13 | -0.37 | -0.36 | -0.36 | -0.36 | -0.36 | -0.35 | -0.35 | -0.35 | -0.35 | -0.35 | -0.35 | -0.35 |
| Genotype | 0.10 | -0.13 | -0.03 | -0.13 | 0.13 | 0.07 | 0.08 | 0.04 | -0.14 | -0.05 | 0.00 | 0.08 | 0.08 | 0.0000 |
| Treatment | -0.02 | -0.04 | -0.11 | -0.08 | 0.06 | 0.02 | 0.09 | 0.18 | 0.13 | -0.03 | -0.05 | 0.01 | 0.05 | 0.03 | 0.0000 |
| Replicate | -0.02 | -0.04 | -0.11 | -0.08 | 0.06 | 0.02 | 0.09 | 0.18 | 0.13 | -0.03 | -0.05 | 0.01 | 0.05 | 0.03 | 0.0000 | 0.0000 |

Note: PH: Plant height; NL: Number of leaves; SL: Stem length; SG: Stem girth; LL: Leaf length; LB: Leaf width; NI: Number of inflorescent; IL: Inflorescent length; IB: Inflorescent width; NB: Number of branches
Notable amongst the molecular marker used for genetic diversity studies, linkage map construction, and marker-assisted selection (MAS) is Microsatellites (Chung et al., 2009; Zhao et al., 2011). All primers used were polymorphic across all the *Amaranthus* genotype with amplified microsatellites loci. This shows a further indication of the ability of these primer pairs to distinguish different genotypes of *Amaranthus* in treatment combinations.

There were variations in major allele frequency, number of amplified microsatellite loci, number of allele and allele diversity. This supported the report of Mallory et al., (2008). Mandal and Das (2002) observed a high degree of genetic diversity when they worked with grain *Amaranthus* using RAPD markers, which was similar to the report of Transue et al. (1994) and Chan and Sun (1997). However, Plaschke et al. (1995) confirmed that a relatively small number of SSRs are sufficient to discriminate closely related bread wheat cultivars. These studies revealed that high levels of genetic differences and similarities can still exist amongst the *Amaranthus* genotypes even in treatment combinations.

As co-dominant and locus specific markers, SSR primer pairs can be used to monitor line uniformity between and within lines of a clone. This information can assist breeders in selecting the appropriate treatment to be applied on *Amaranthus* so as to obtain maximum yield and equally maintain genetic variability. The SSR markers were found to be suitable for genetic mapping studies, which will assist efforts towards marker assisted selection (MAS) in *Amaranthus*. The results from this study also clearly demonstrated the effectiveness of the SSR markers for fingerprinting *Amaranthus* spp. genotype as a means of agronomic improvement.

Molecular markers allowed us to estimate the overall genetic similarities and differences in *Amaranthus* and simultaneously to reveal molecular-based genetic relationships. Cluster analysis and dendogram, indicates that cluster groups consist of genotypes from different treatment combinations. The cluster diagram among the treated *Amaranthus* genotype showed that there were genetic similarities and differences. Thus, high inter- and intraspecies variability exists even among genotypes (Mosyakin and Robertson, 1996; Štefínová et al., 2014). Moreover, genotypes with different treatments were found in different clusters. Such wide adaptability has been attributed to similarity in selection history, requirements, cultivations and developmental traits. The relationships that exist among the genotypes in the clusters show that there were genetic similarities which were similarly reported by Bamgbegbin et al. (2016). Identification and conservation of germplasm are necessary for maintaining genetic diversity, studying local genetic material in order to choose ecotypes having high nutritional interest in their place of origin (Perez-Gonzalez, 2001). Moreover, the genetic information obtained from this study could aid the design of useful and appropriate tool for selecting representative genotypes and effectively managing *Amaranthus* germplasm breeding programs.

**3 Conclusions and Recommendation**

ASAAC001 primer detected the highest polymorphism, allele and gene diversity compared to other primers. This suggests that this primer could be used for further molecular breeding of other vegetables. Since *Amaranthus* has been confirmed as an under-utilized crop, this will prevent its gradual loss of genetic diversity.

The establishment of genetic similarity and phylogenetic relationship among *Amaranthus* genotypes in treatment combinations of bioinoculants could further be an efficient means of introducing novel variability into *Amaranthus* gene pool. NGB01271 genotype could be selected, while GM, AMF and combinations of AMF and GM could be recommended for crop improvement breeding programs with a view to adopt Integrated Nutrient Management Approach (INMA) for yield improvement and proper documentation of germplasm conservation of *Amaranthus* spp for future diversity studies.

**4 Materials and Methods**

*4.1 Germplasm collections, study location and plant samples*

The seeds of the five genotypes of *Amaranthus* spp. evaluated in this research study were collected from the National Centre for Genetic Resource and Biotechnology (NACGRAB), Moor Plantation, Ibadan, Nigeria. The genotypes evaluated were: NGB01271, NGB01644, NGB01234, NGB01613 and NGB01662. The molecular studies were carried out in the Laboratory of Bioscience Unit of International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Fresh leaves samples from the five genotypes of *Amaranthus* were collected very early in
the morning by 8:00 am after two weeks of planting. The collected leaf samples were kept in a cold environment at a temperature of 0°C so as to prevent denaturation of their DNA contents. A total of five leaf samples were randomly collected per treatment from the three replicates for DNA extraction. Twenty leaf samples were collected altogether from each block. From the whole experimental set-up, a total of one hundred leaf samples were collected altogether and lyophilized at -80°C.

4.2 Planting procedure
The field Experiment was a complete Randomized Design which was properly spaced at 22 cm between treatment and 65 cm between blocks. The genotypes were subjected to treatments as follow, T1 = uninoculated (control), T2 = 5 g AMF and T3 = 5 g GM while T4 had a combination of 2.5 g AMF and 2.5 g GM, replicated thrice. The treatments comprised of Arbuscular Mycorrhiza Fungus (AMF), Green Manure (Leucaena leucocephala) and combinations of Arbuscular Mycorrhiza Fungus (Glomus clarum) and Green Manure (Leucaena leucocephala). These treatments were applied after two weeks of transplanting. The set-ups were moderately watered to resist drought and enhance development.

4.3 DNA extraction and quantification
Extraction of DNA from fresh leaf sample was the first step using molecular marker and primers. The DNA was extracted from fresh and lyophilized young leaf samples of Amaranthus in order to obtain good quality DNA. This DNA extraction was carried out according to Dellaporta et al., (1983) protocol modified in sodium dodecyl sulphate (SDS) extraction buffer. One gram of fresh young leaves of Amaranthus was grinded using mortar and pestle followed by the addition of one litre of extraction buffer. The extraction buffer consisted of isopropanol, 10 mg/ml RNASE A, β-mercaptoethanol, 1 % PVP (1 g of 100 ml), 5M NaCl (292.2 g in 1 000 L), 1M Tris (121.4 in 1 000 ml, PH 8), 0.5 M EDTA (146.12 g in 1000 L, PH8), 20% SDS (200 g SDS + 800 ml of double distilled water), 70% ethanol (70 ml of ethanol in 100 ml of double distilled water), 25 ml CIA (24 ml chloroform + 1 ml Isoamyl Alcohol), 5 M Potassium Acetate (490.7 in 1,000 ml) stirred at 4°C, Low salt TE buffer (10 ml 1 M Tris HCL, 2 ml EDTA, 950 ml of double distilled water and adjusted to PH to 8).

One litre of this buffer was prepared using, 100 ml 1 M Tris HCL + 50 ml 0.5 M EDTA + 100 ml 5 M NaCl + 1% PVP.

DNA extraction protocols involved grinding or digesting cellular constituents in order to release the content. Detergents such as sodium dodecyl sulphate (SDS), Cetyl trimethylammonium bromide (CTAB) was used for the removal of membranes lipids. When the DNA is released, it is protected from endogenous nucleases by the inclusion of EDTA in the extraction buffer which is necessity for the chelating magnesium ions that are a significant co-factor for nucleases. The eppendorf tubes were thoroughly mixed and incubated at 65°C for 60 minutes in a water bath. 5 ml of 5 M potassium acetate (PH 5.5) was added to each of the tubes and kept for 20 minutes at 4°C in ice. The mixture was centrifuge at 15,000 rpm for 15 minutes and 10 ml of ice cold isopropanol was added to the supernatant and kept for incubation at 4°C for 30 minutes. The solutions were centrifuged at 15,000 rpm for 15 minutes and the pellets were dissolved in sterile double distilled water. The DNA extract usually contains sizeable amount of RNA, proteins, polysaccharides, tannins and pigments that may interfere with the extracted DNA. An addition of protein degrading enzymes known as proteinase-K is use to remove the proteins, followed by denaturation at 65°C and precipitation using chloroform and Isoamyl alcohol. Also, RNAs are normally removed using RNA degrading enzyme known as RNase A. The DNA solutions was transferred to 2 ml eppendorf tube and treated with RNAses (10 mg/ml) for 1hr at 37°C and 1ml of chloroform: isoamyl alcohol (24:1) was added and Centrifuged at 12,000 rpm for 15 minutes. Though, Polysaccharide-like contaminants are, more tasking to remove. However, the combinations of NaCl and CTAB have been observed to remove polysaccharides (Murray and Thompson, 1980; Paterson et al., 1993). Moreover, some protocols replace NaCl with KCl (Thompson and Henry, 1995). Since DNA will be released along with other compounds like lipids, proteins, carbohydrates or phenols. It needs to be separated from the other compounds by centrifugation. The DNA in the aqueous phase will then be transferred into new eppendorf tubes without disturbing the interphase and ice cold ethanol was added to precipitated the DNA in salt solution (e.g. sodium acetate) or alcohol (100%
isopropanol or ethanol), re-dissolved in sterile water or buffer. The precipitate was centrifuged at 12,000 rpm for 10 minutes and the supernatant discarded. The pellet was washed in 70% ethanol; air dried and finally dissolved 100 μl of sterile double distilled water. The determination of DNA concentration extracted needs to be measured using 1% agarose gel electrophoresis and detected using UV illuminator or spectrophotometer. Agarose gel checks whether the DNA is degraded or not but estimating DNA concentration by visually comparing band intensities of the extracted DNA with a molecular ladder of known concentration is too subjective. Spectrophotometer measures the intensity of absorbance of DNA solution at 260 nm wavelength, and also indicates the presence of protein contaminants but does not determine whether the DNA was degraded or not.

The DNA quantification was done to ascertain the quality and quantity of DNA extracted from the plant samples. Purity of the DNA in the samples, dissolved in TE buffer was analyzed by checking the absorbance ratios at 260/280 nm on nanodrop spectrophotometer followed by determination of concentration.

### 4.4 Primers design of simple sequence repeat (SSR) marker

Primers flanking each unique microsatellite were designed using the web-based computer program Primer3 version 2.0 (Rozen and Skaletsky, 2000) according to the program’s default parameters, with the following exceptions: preferred product size range equal to 150-200 base pairs; melting temperature differences in forward and reverse primers of no more than 1°C; and max poly-X (maximum allowable length of a mononucleotide repeat) of three. These primers were designed and used for the flanking regions of the SSRs by Melanie Ann Mallory in 2007. Oligonucleotide primers were synthesized by Inquaba Biotech, Ibadan. Primer pairs were assigned names based on their repeat motif that is AS = *Amaranthus spp*, AAC = motif type while clone ID were 001, 005, 006 and 011 which resulted to a total of four primers having both forward and reverse sequence were selected from the designed by Melanie Ann Mallory (2007) (Table 11).

| PRIMERS  | Primary motif | Forward primer (5’-3’): | Reverse primer (5’-3’): |
|----------|---------------|-------------------------|------------------------|
| ASAAC001 | (CAA)₆        | GGGTATGAAATGTTCCGGAAT  | CTTGTCCTTACATCCCCATACTGTTG |
| ASAAC005 | (CAA)₅        | CCATCATTGCACCACAGAAGA  | TGTCGACGGTTAGTCTCG      |
| ASAAC006 | (GTT)₃(GCT)₂(GTT)₂ | TTGAAAGATCGATGCCACAC | CGTTCAAGAAAGCATGTGGA    |
| ASAAC011 | (GTT)₅        | CCGTCTGTGCTGTATTGAGG   | GGCCACTGGGTATTCTCT      |

### 4.5 Polymerase chain reaction (PCR), amplification conditions and amplification of DNA product

PCR reactions for SSRs was carried out in the presence of forward and reverse primers that anneal at the 5’ and 3’ ends of the template DNA respectively. PCR fragments are usually separated on polyacrylamide gels in combination with AgNO₃ staining, autoradiography or fluorescent detection systems. Agarose gels (usually 3%) with EtBr can also be used when differences in allele size among samples is larger than 10 bp.

The amplification conditions was: an initial step of denaturation for 5 minute at 94°C followed by 44 cycles each consisting of a denaturation step of 15 seconds at 94°C, an annealing step of 20 seconds at 65°C and an extension step of 30 seconds at 72°C. Seven minutes will be given after the last cycle to the extension step at 72°C to ensure the completion of the primer extension reaction followed by hold temperature at 10°C lasting for infinity. The PCR products were separated on 5% polyacrylamide gel at 80 volt, 300 mA 60 Watt for 1 hour 30 mins. It was then visualized and photographed using silver staining under UV transilluminator (Zhang et al., 2002).

Amplification of microsatellite loci were carried out in 10 μl PCR reactions containing 3.0 ul of 100 ng/ul total genomic DNA, 0.5 μl of 5pMol of forward and reverse primers each, 0.8 ul DMSO, 0.4 ul of 50 mM MgCl₂ 0.8 ul of 2.5 Mm DNTPs, 2.9 ul H₂O and 0.1 ul of Taq DNA polymerase using Master Mix. Amplification was performed using an appendorf thermocycler.

### 4.6 Polyacrylamide gel electrophoresis (PAGE)

The reagents used was prepared from a mixture of the following; 40 ml of water, 25 ml of TBE, 7.5 ml of polyacrylamine, 50 μl of Terred and 500 μl of APS was added last.
The procedures included the glass comb and rubbers were washed in order to dry. The glass plate and spacer were wiped with 70% ethanol. The glass plate was lined with rubber and the spacer was placed between both spacer. The plates were carefully clipped together (the blue clip is used to clip the plates together, while the white clip is used to clip the electrophoretic tank.

When the glass tank was well arranged and positioned, the mixed reagent was poured into it until it gets to the brim. It is allowed for some minutes to gel. When it gels, it is then inserted into the gel tray where the DNA samples are introduced using a staining dyes alongside the 1 kb plus ladder gene ruler from Thermos scientific for formation of bands. The gel was then brought out after 1 hour 30 mins and loaded into the chamber for viewing using the UV trans illuminator.

4.7 Determination of growth and yield characters
Data taken on morphological characters included plant height (cm), number of leaves, stem length (cm), stem girth (cm), and leaf area (cm²). At maturity period, data were also collected on the quantitative and qualitative flower characters which included; number of inflorescence, inflorescence width (cm), inflorescence length (cm) and number of branches. The Genotypic variance, phenotypic variance and Heritability were determined using the following formula:

\[
\text{Genotypic variance} = \frac{\text{Genotype MS - Error MS}}{\text{Replicate}}
\]

\[
\text{Heritability} = \frac{\text{Genotypic Variance}}{\text{Phenotypic Variance}}
\]

\[
\text{Phenotypic Variance} = \text{Genotypic Variance} + \text{Error MS}
\]

4.8 Statistical analysis
The data on growth and yield characters were subjected to Analysis of Variance (ANOVA) using SAS 9.3 software while the means were separated by DMRT.

Molecular data generated was subjected to molecular analysis in order to generate information on Total gene diversity, Gene diversity per locus using PopGene Version 1.32 (Yeh and Boyle, 1999) and Powermarker V 3.25 (Liu and Muse, 2005). Nei's coefficient of gene variation and genetic distance was calculated according to Nei (1972). These values were used to generate dendogram using Unweighted Pair Group Method with Arithmetic Average (UPGMA) cluster analysis as described by Sneath and Sokal (1973) to reveal phenetic representations of genetic relationship among the treated *Amaranthus* spp.

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