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Development of simple *in-vitro* protocol for screening low soil nitrogen-efficient maize lines

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ABSTRACT

In order to fast track and compliment conventional maize breeding programme designed for low nitrogen (N) environments, seeds of eight low soil N-tolerant maize lines were sown in the field under no nitrogen and 40 kgN/ha for *in vivo* evaluation with a view to develop simple protocols for screening maize lines for low **nitrogen-efficient** through tissue culture technique. Maize plants used for tissue culture (*in vitro*) were selfed, cobs were harvested fourteen days after pollination before the kernels were disinfected. The immature embryos were excised and cultured on modified Murashige and Skoog basal medium (MS) containing 100% Nitrogen (1650 mg/l), 50% Nitrogen (825 mg/l), 25% Nitrogen (412.5 mg/l), 0% Nitrogen of normal ammonium nitrate in MS salt formulation. Results obtained revealed three lines (LNTP-YC6, TZPB Prol C3 and SW5-OB-BPR-1) had higher grain yield than other maize lines under no fertilizer (0 kgN/ha) and 40 kgN/ha fertilizer applications ($P = 0.01$) while two lines (TZPB Prol C3 and LAPOSTA SEQUIA C6) performed better than others with or without ammonium nitrate under *in-vitro* condition ($P = 0.01$). TZPB Prol C3 was consistent in both *in-vitro* and *in-vivo* conditions. Also, MS medium without ammonium nitrate appeared to be more suitable for screening for low N-tolerant maize genotypes.

1. Introduction

Maize (*Zea mays* L.) is a major cereal crop for livestock feed, human nutrition and important raw materials for several agro-based industries in Nigeria [1]. It is the third most important cereal crop after wheat and rice in terms of production in the world [2]. Nigeria produces 43% of all maize grown in West Africa [3].

Nitrogen (N) is one of the major limiting essential nutrients to maize production [4,5]. Although, increase in maize production can be achieved through increased levels of fertilizer application. However, non-availability of these fertilizer and high cost sometime constitute limiting factors to achieving increased production [6]. In Nigeria, like many tropical African countries, funding of agricultural research that are input based appear expensive to many Research Institutes due to dwindling research grant that can accommodate the associated cost. Screening maize lines tolerant to nitrogen stress and provision of nitrogen fertilizer, therefore, call for fast and less expensive method for effective selection of maize genotypes that will reduce time and cost when developing N-tolerant maize genotypes.

Mineral nutrient (N) is one of the most important and basic components of plant tissue culture. The major source of N is usually nitrate, ammonium and amino acids [7]. Growth and morphogenesis of plant tissues under *in vitro* conditions are largely influenced by the composition of the culture medium. Murashige and Skoog basal (MS) medium is the most used tissue culture medium, because it contains higher amount of nitrate (N) than other salt formations. It also, contains all the nutrients required for the normal growth and development of plants under aseptic and controlled environment. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium [8–10].

Most of the farm lands available for cultivation of maize by resource-limited rural farmers in Nigeria are depleted of N due to monocropping and non-availability of fertilizer. This could be mitigated through selection for low N maize genotype by using biotechnological approach within a short time. Therefore, tissue culture offers opportunities to study cellular level responses of plants to nitrogen stress and possibly identify cell lines that differ in responses to variation in nitrogen levels of soil. It also provides the benefit of developing fast techniques to evaluate and screen potentially tolerant germplasm for

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low-nitrogen tolerance. Therefore, the objective of this study is to develop simple protocols for screening maize lines for low Nitrogen tolerance through tissue culture technique with a view to fast track and compliment conventional maize breeding programme designed for low nitrogen environments.

2. Materials and methods

2.1. Seed collection and preparation

Seeds (200 g) of eight nitrogen-tolerant maize lines (ERUWA LOCAL-2-1, SW5-OB BPR-1, ARTP8/SW6-OB-1, BR99 TZL Comp.4 DMSRSR, LNTP-Y C6, SINT MARZOCA LARGA, LA POSTA SEQUIA C6 and TZPB Prol C3) were obtained from Maize Gene Bank of Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, Ibadan, Nigeria. Seeds (200 g) from each maize line were divided into two; half was used for field (*in vivo*) screening while the other half was used for *in vitro* screening.

2.2. In-vivo screening of low soil nitrogen-tolerant maize lines

Field experiment was conducted at the Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, Ibadan, Nigeria, Latitude $7^0$22.5'N and Longitude $3^0$50.5'E in the growing seasons of year 2011 and 2012. The field has been under continuous cultivation of maize for more than 15 years. Physico-chemical analysis of the field conducted indicated that the soil pH was 6.09, Organic matter 1.33%, Organic N 0.096%, and available P 7.36 (ppm), Sand 75.9% Silt 15.5% and Clay 7.8% [11]. The seeds were sown in plot size of 4.5 m x 4 m at 75 cm × 50 cm at two seeds per hill in two rows, laid out in a Randomized Complete Block Design (RCBD) with three replicates in split plots design. Low fertilizer concentration of 40 kgN/ha was applied four weeks after planting as side dressing to one part of the plot while fertilizer was not applied to the other corresponding part. The 40 kgN/ha was applied to identify promising genotype(s) that can produce sizeable cobs at fertilizer rate lower than the recommended dosage (60 kgN/ha) which can eventually be recommended to farmers in Nigeria. All agronomic practices needed for maize production were carried out. At maturity, all the cobs in the plot were harvested, shelled and dried to determine the grain yield per plot and grain yield/ha according to Olakojo and Olaoye, [12].

2.3. In-vitro screening of low soil nitrogen-tolerant maize lines

Seeds of the eight maize lines were grown on the field while plants to be used for *in-vitro* screening were selfed to prevent contamination. Fourteen days after pollination (14 DAP), the immature cobs were harvested and the kernels were removed with scapel [13]. The kernels were disinfected in 70% methylated spirit, 0.1% and 0.2% mercuric chloride respectively and rinsed in three changes of sterile distilled water and immature maize embryos were excised. Embryogenic calli were induced to select for those tolerant to low levels of nitrogen by culturing excised immature maize embryos on modified Murashige and Skoog (MS) basal medium [8] containing four concentrations: MS medium + 100% Nitrogen (1650 mg/l), MS medium + 50% Nitrogen (825 mg/l), MS medium + 25% Nitrogen (412.5 mg/l), MS medium + 0% Nitrogen (nil) of normal ammonium nitrate in MS salt formulation (nitrogen source) and each treatment was further supplemented with 30 g/l sucrose and 8 g/l agar with addition of phytohormone (auxin) 2 mg/l of 2,4-D in culture medium. Since it has been established and reported by several Scientists that the presence of 2 mg L^{-1} of 2,4-D in culture medium was critical for callus induction and embryogenic callus formation from immature embryos [9,14,15,16], 2 mg/l of 2,4-D was used in the culture medium in this study to induce callus from the immature embryo (14 DAP). The cultures were maintained for four weeks after which the following data were collected and evaluated; the degree of callus, shoot and root formation respectively was recorded as the fraction of the cultured explant on which callus had been induced on a scale of: 0: no callus, 1: lowest (up to 20%), 2: lower (21–40%), 3: medium (41–60%), 4: high (61–80%) and 5: highest (81–100%) [13].

Table 1
Mean square values for grain yield (t/ha) of eight maize lines evaluated under Nitrogen levels environments.

SoV	df	MS
Genotype(G)	7	0.335**
Nitrogen levels(N)	1	0.574**
Year(Y)	1	0.233**
G x N	7	0.034**
G x Y	7	0.105**
N x Y	1	0.020**
G x N x Y	7	0.009**
Error	32	0.01

SoV: Source of variation; df: degree of freedom.
** Significant at $p < 0.01$.

2.4. Data analysis

Analysis of variance (ANOVA) was performed on the pooled data collected using Statistical Tool for Agricultural Research [18] software for *in vivo* and *in vitro* data respectively. Difference between treatments mean was separated by the Duncan Multiple Range test (DMRT) at 5% and 1% levels of probability.

3. Results and discussion

3.1. Performance maize lines under field screening

Genotype, nitrogen levels and years of evaluation had high significant effect on grain yield of maize lines ($p = 0.01$) (Table 1). Also, the interactions between the various components (G x N, G x Y, N x Y and G x N x Y) had highly significant effect on grain yield of maize lines ($p = 0.01$) in this study (Table 1). This indicates that maize grain yield is dependent on many factors like genetic make-up of the genotypes, fertility levels of the testing environments and years of evaluation of the genotypes and their interactions. This corroborates the findings of Anjorin, [11] who reported that genotype, nitrogen levels and years of evaluation greatly and significantly influenced grain yield of five open pollinated maize varieties tested on native soil fertility and N fertilized soil environments in 2010 and 2011 in Moor Plantation, Ibadan, Nigeria.

The maize lines evaluated in this study had average grain yield of 2.33t/ha and 2.24t/ha in 2011 and 2012 respectively under no fertilizer (0kgN/ha) application while average grain yield of 2.55t/ha and 2.39t/ha were recorded under 40kgN/ha fertilizer application in 2011 and 2012 respectively (Table 2). Yield of the eight maize lines treated with 40kgN/ha (2.47t/ha) were significantly higher than those without nitrogen application from pooled data (2.29 kg/ha) (Table 2). Genotypic variation was observed among the eight maize lines evaluated; LNTP-YC6 and TZPBProl C3 were found to give the highest yield under no fertilizer (2.64 and 2.45t/ha respectively) and 2.73t/ha for both N-levels (Table 2).

Several authors have reported that different genotypes performed differently across different soil fertility levels [19,20,11]. This corroborates the work of Ajala et al., [21] who evaluated LNTP-Y C4 and TZBP Prol grain yield at Mokwa and Zaria in Nigeria in 2002 and 2003 at three N-levels and reported that LNTP-Y C4 had higher grain yield of 1802 kg/ha than the 3 checks used in the experiment (Oba Super 2, TZB-SR and Oba Super 1). This result is an indication that there was persistent year effect on the observed genotypic variability while