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Exploiting polyploidy in *Pennisetum Purpureum* to increase forage yield, feed value and tolerance to diseases

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ABSTRACT

Napier grass (Pennisetum purpureum) is a key forage crop in Kenya. However, its yield and quality are often hindered by headsmut and stunt disease. Genetic improvement through mutation breeding, particularly using colchicine to induce polyploidy, offers a potential solution for improving Napier grass.The experiments were carried out as a factorial experiment in a complete random design (CRD). This study aimed to evaluate the response of embryogenic calli to different colchicine concentrations (0, 0.05, 0.1, and 0.2%) over 24, 48, and 72 hours duration to induce polyploidy in *South african* and *Bana* napier grass germplasms. The most suitable media for shoot regeneration was Murashige and Skoog (MS) medium supplemented with 0.2 mgL-1 Benzyl Adenine (BAP), 0.1mgL-1 dichlorophenoxyacetic acid (2, 4-D) and 0.1mgL-1 Indole-3-Butyric Acid (IBA) while media with 1mgL-1IBA, 1mgL-1 2, 4-D and 0.5mgL-1 BAP was more suitable in inducing embryogenic calli in all genotypes. Chromosome doubling was confirmed through chromosome counting and stomatal size, and number. Notably, we recommend use of flow cytometry to confirm ploidy level. Results showed that a 0.1% colchicine concentration with a 48-hour treatment was most effective for producing mutant plantlets, while higher concentrations were toxic. Significant genetic and agronomic variations were observed between the mutants and controls, indicating that the selected mutants are valuable genetic resources and recommended for characterization across representative agro-ecologies for large-scale production and used in *Penniseturm purpureum* breeding programs.

Introduction

Livestock plays a crucial role in food security, income, and employment, with global meat and milk demand expected to double by 2050(Herrero et al., 2013). In Kenya, smallholder farmers are key contributors to beef and dairy production, Napier grass (*Pennisetum purpureum*) is the primary livestock feed in Kenya, particularly in zero-grazing and semi-intensive systems (Muyekho et al., 2003; Lukuyu et al., 2011). However, continued contribution of napier grass to the livelihoods of the small-scale farmers is threatened by low growth vigor, low biomass and low feed value due to inferior germplasm (Jones et al., 2004).

Therefore, there is a need to enhance genetic diversity of pasture and forage to meet growing demand, and this is a key strategy for improving food crops resulting to enhanced plant performance (Ardabili and Zakaria, 2015). In light of the above background, this study aimed to investigate the response of embryogenic calli to different colchicine concentrations in inducing polyploidy for regeneration and selection of novel napier grass mutants.

Materials and methods

Experimental site and Plant materials

The experiment was conducted as a factorial with three factors: four levels of colchicine concentration (C0(0%), C1(0.05%), C3(0.2%) and C3(0.2%)), three levels of exposure period (T1 24, T2 48 and T3 72) and two napier grass germplasm (V1 *South africa* and V2 *Bana grass* resulting to 24 treatment combinations, that were replicated thrice using a factorial completely randomized design (CRD) resulting to 96 experimental unit. However, a pre-experiment needed to be conducted to carryout somatic embryogenesis to come up with explants to be used in the next stage of study which is colchicine treatment and acclimatization in the greenhouse. This pre-experiment was conducted as a factorial experiment in a completely randomized design(CRD) having three factors; 3 growth hormone for callus induction, 3 growth hormone for shoot regeneration and rooting and 2 germplasms of napier grass. This resulted to 18 treatment combination that were replicated six times. This resulted to 108 experimental units where the best explant that formed embryogenic calli were selected for the next stage of study. The following callus induction medium was used; GM0 as a comparative control in a hormone free media, GM1 (MS media supplemented with 0.3mg/L-1 BAP, 0.5mg/L-1 2,4-D and 0.5mg/L-1 IBA) and GM2 (MS media supplemented with 0.5mg/L-1 BAP, 1.0mg/L-1 2,4-D and 1.0mg/L-1 IBA). For Shoot regeneration, the following medium was used; SRM0 as a comparative control in a hormone free medium, SRM1 (MS media supplemented with 1mg/L-1 BAP, 0.25mg/L-1 2,4-D and 0.25mg/L-1 IBA and SRM2 (MS media supplemented with 2mg/L-1 BAP, 0.5mg/L-1 2,4-D and 0.5mg/L-1 IBA.

Embryogenic calli formation and shoot regeneration

Shoot tips from the two Napier grass genotypes were surface sterilized and prepared for tissue culture. Callus induction and shoot regeneration were achieved using Murashige and Skoog (MS) medium with specific plant growth hormones, while rooting involved transferring shoots to a medium supplemented with NAA, 2,4-D, and ascorbic acid.

Treatment with Colchicine

The impact of colchicine concentrations and treatment duration on explants survival and polyploidy induction was examined as a factorial experiment in a CRD with three replications. Explants were exposed to treatment after 134 days of culture by immersing them in filtered- sterilized colchicine solution for the designated times as stated earlier, then rinsed three times with sterile distilled water. Explants were then cultured on shoot regeneration media Ms supplemented with various concentrations of BAP (0, 1, 2 mg/L), 2, 4-D (0, 0.25, 0.05 mg/L), and IBA (0, 0.25, 0.05 mg/L). For rooting plantlets, elongated shoots were transferred to rooting medium as stated earlier.

Evaluation of induced mutants to determine ploidy level

Two months after transferring them to the greenhouse after treatment with colchicine, plants that regenerated were subjected to screening for confirmation of polyploid by measuring stomata number and size, chromosome counting and genomic DNA of mutant plants *viz* their progenitors.

Chromosome number by Karyotyping

Chromosome counting was done through karyotyping by treating leaf samples with 3% chromic acid, 20%formaldehyde, and 6% aceto-carmine, before observing them under a light microscope at X80 and X100 magnification

Stomata size and Number

Stomatal density and size were measured by applying nail polish on 35 day old leaves measuring 0.2cm^2 and counting stomata under a microscope at X20 and X40 (Yu et at., 2009)

Phenotypic evaluations of induced mutants

Phenotypic evaluations of the induced mutants included weekly counting of tiller number and measuring plant height, and stem diameter, with stem diameter measured 10 cm from the base and height recorded from the ground to the highest point.

Results

Response of genotypes to tissue culture

The analysis revealed significant genotype effect on callus formation, necrosis, and embryogenic callus formation. After 4 weeks, all genotypes formed 97.5% calli, with no significant differences in germplasm, and no embryogenic callus formed. After week 7 and 8, The percentage of embryogenic callI after 8weeks was 68.9% and 66.2% on germplasm V1 and V2 on media with 1.0mgL-1 IBA, 1.0mgL-1 2,4-D and 0.5mgL-1 BAP while genotypes V1 and V2 on media with 0.5mgL-1 IBA, 0.5mgL-1 2,4-D and 0.3mgL-1 BAP formed 31.1% and 33.8% calli . Explants cultured on hormonal free media did not produce any sign of growth of callus or embryogenic callus, they were also 100% necrotic. V1 had the highest necrosis after 4 weeks 14.9% and 8 weeks 12.2% while V2 had the lowest case after 4 weeks 9% and 8 weeks 4% necrosis. (Table.1)

Table 1. Effects of different growth hormone combination on embryogenic calli induction.

Growth Media	Genotype	No.of Calli formed/% (4weeks)	No. of Necrosis formed/% (4weeks)	No. of embryogenic calli formed/% (4Weeks)	No. of embryogenic calli formed/% (8weeks)	No. of Necrosis formed/% (8weeks)
GM0	V1	0(0)	96(100)^{a}	0(0)	0(0)	0(0)
	V2	0(0)	74(100) ^{ab}	0(0)	0(0)	0(0)
GM1	V1	65(87.83)^{c}	11(14.86)^{b}	0(0)	25(33.783)^{c}	9(12.162)^{a}
	V2	63(85.13)^{d}	9(12.16)^{d}	0(0)	23(31.081)^{d}	4(5.405)^{d}
GM2	V1	68(91.89)^{b}	9(12.162)^{d}	0(0)	51(68.919)^{a}	6(8.108)^{c}
	V2	69(93.24)^{a}	10(13.514^{c}	0(0)	49(66.216)^{ab}	8(6.757)^{b}

Mean levels with ^{abcd} different numbers following each value within a column demonstrate significant differences by Tukeys Test ($p \leqslant 0.05$)

Shoot and Root regeneration

Growth of shoot was initiated after 134 days in most media, with regeneration significantly influenced by different hormone combinations. Hormone- free media resulted in no growth and 100% necrosis. The most effective media for shoot regeneration was Ms supplemented with 0.05mg/L IBA, 0.05mg/L 2,4-D, and 2mg/L BAP, achieved 43 and 40% shooting (Table 2). After 14 days, the putative mutants were transferred to the greenhouse, where rooting was successfully induced using MS medium with 1mg/L NAA and 150mg/L ascorbic acid (Table 2, Fig 1)

Table 2. Growth hormone combination effects on shoot induction derived from shoot tillers of two genotypes of *Penniseturm purpureum*

Growth Media	Genotype	No. of regenerated clumps (%)	No. of regenerated shoots (%)
SRM0	VI	0(0)^{ns}	0(0)^{ns}
	V2	0(0)^{ns}	0(0)^{ns}
SRM1	V1	16(21.6)^{c}	15(20.27)^{c}
	V2	14(19)^{d}	11(14.86)^{d}
SRM2	V1	37(50)^{a}	32(43.24)^{b}
	V2	32(43)^{b}	30(40.54)^{a}

Mean levels with ^{abc} different numbers following each value within a column demonstrate significant differences ($p \leq 0.05$). SRM0 is explants established in hormone free media. SRM1 is MS media supplemented with 1.0mg/L-1 BAP, 0.25MG/L-1 2,4-D and 0.25mg/L-1 IBA. SRM2 is MS media supplemented with 2.0mg/L-1 BAP, 1.0mg/L-1 2,4-D and 1.0mg/L-1 IBA

Fig.1 Regeneration of two genotypes of penniseturm purpureum through somatic embryogenesis. a) Embryogenic callus in MS media supplemented with 0.5mg/L-1 BAP, 1.0MG/L-1 2,4-D and 1.0mg/L-1 IBA . b) Sprouting embryos in different growth stage in calli treated with colchicine. c) Shoot regeneration and root induction with MS medium supplemented with 0.05mgL-1 IBA, 0.05mgL-1 2,4-D and 2mgL-1 BAP and transferred to media supplemented with NAA 1mgL-1 and 150mgL-1 Ascorbic acidl. d) Regenerated synthetic induced mutants with their progenitors after being potted and transferred in the greenhouse

Effect of different colchicine concentrations on survival of explants and ploidy induction

Explants from two germplasm were treated with different colchicine concentrations on solid media. The percentage (%) survival rate differed depending on colchicine concentration, exposure duration and

temperature. Higher concentrations and longer exposure time led to severe toxicity and reduce callus survival, however, it increased octoploidy induction. The optimal condition for inducing polyploidy was 0.1% colchicine with a 48 hours exposure, resulting to 48% polyploidy. Putative mutants exhibited shorter height, increased tillering, and reduced stem diameter compared to their progenitors. Chromosome counting and stomata size and number confirmed successful polyploidy induction (Fig 2)

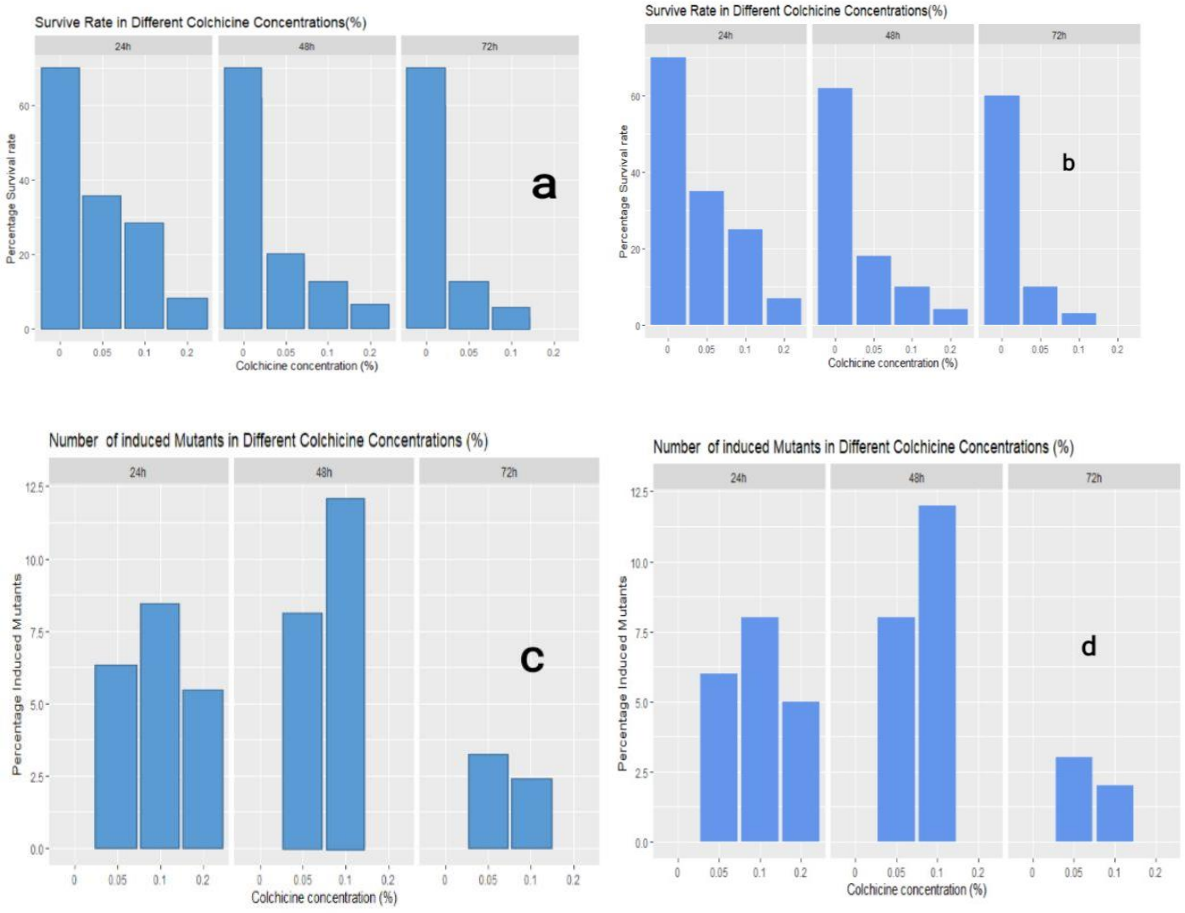

Fig 2 Different letters shows differences at ($p \leqslant 0.05$)
ns Non significance

(a) Colchicine concentrations; C0=0,C1=0.05, C2=0.1 and C2=0.2%
(b) Scores as 1-3 shows variation in survival rate after treatment where 1=5-25%, 2=26-45% and 3=46-70%

Score as abc shows variation in No. of induced mutant where a=2-5 %, b=6-8% and c=12%

Phenotypic effect on leaf characteristics

Induced mutants stomata size and number was significantly different from their progenitors. Mutants exhibited larger stomata with stomatal length of 20 or more (>120um) than their progenitors, however, they had lower stomatals density (Table 3, Fig 3). Similarly, analysis of variance detected high significance in tillering ability after 6 weeks of establishment in the greenhouse where induced mutants were the first to produce tillers but after 8 weeks, there was a high significance in number of tillers between induced mutants with their progenitors (Table 3,Fig. 4). Induced plants had slow growth rate as a result of chromosome

abbreviation (deletion, duplication, inversion and translocation), physiological and toxic effect which presumably reduced cell survival (Table 3, Fig. 4). The mean stem diameter of synthetic induced mutants was slightly smaller compared to their progenitors (Table 3, Fig 4)

Table 3. Quantitative and qualitative characteristics in *Penniseturm purpureum* induced mutant's.

Germplasm	stomata number (per mm^2)	Stomata size (mm^2)	Plant height (cm)	No. of Tillers after 6 weeks	No. of Tillers after 8 weeks	Stem diameter after 8 weeks (cm)
C0T0V1	21^{a}	122.54^{c}	190.23^{a}	0^{c}	2^{cd}	5.2^{a}
C0T0V2	19^{ab}	120.65^{cd}	145.31^{c}	0^{c}	5^{b}	4.7^{ab}
C2T2V1	11^{c}	162.44^{a}	123.86^{d}	2^{ab}	3^{c}	4.6^{bc}
C3TIV2	12^{d}	158.45^{b}	170.18^{b}	3^{a}	6^{a}	4.5^{bc}

t-tests were performed between compiled mean from two control lines and those from synthetic induced mutants of each germplasm. Mean levels with ^{abcd} different letters following each value within a column shows significant differences ($p \leq 0.05$).

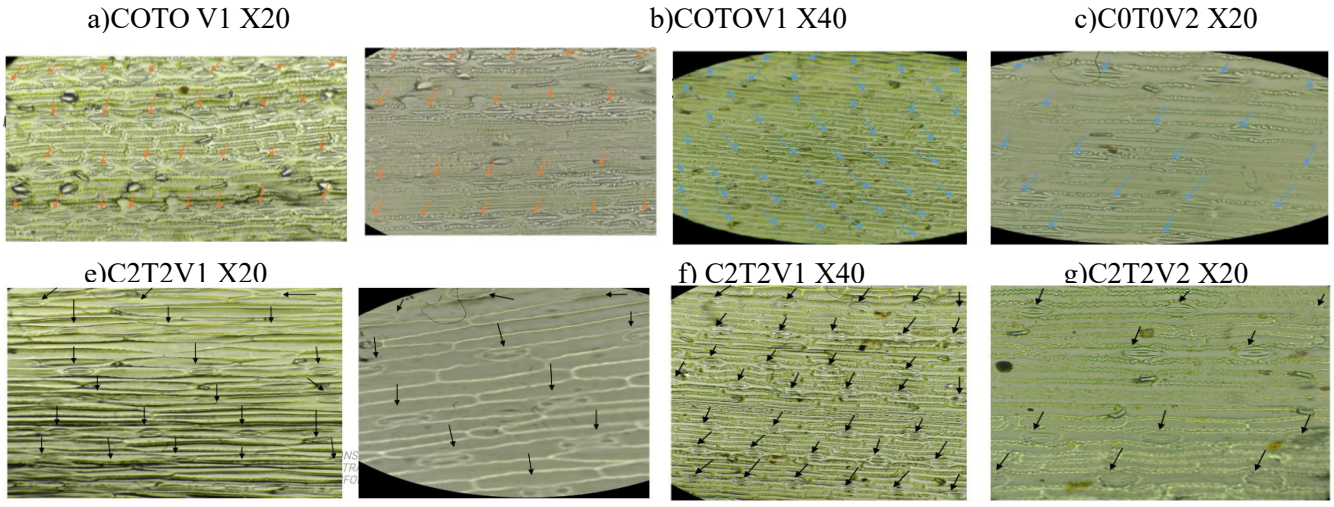

Fig 3 Difference in stomata size and number between synthetic induced mutant and their progenitors at x20 and x40 magnification. Blue and orange arrow points at progenitors stomata while Black arrow points at putative induced mutants.

Fig.6 Chromosome number of Penniseturm purpureum visualized under a light maicroscope at X100

(a) COTOV1 progenitor(2n=4x=28).
(b) C2T2V1 synthetic induced mutant (2n=8x=56
(c) COTOV2 progenitor (2n=4x=28)
(d) C2T2V2 synthetic induced mutant (2n=8x=56

* Measurements of Some chromosomes number were diverse among counts, possibly due to overlaying chromosomes

Fig.4 Abnormalities shown by synthetic polyploidy after treatment with colchicine. A,B and C shows albino plants and leaf chlorosis abnormalities two weeks after treatment with 0.1% colchicine concentration and 48h duration, and 0.2% with 24h and 48h duration of exposure. E, F G and H shows abnormalities several weeks after establishment. E shows chromosome incompatibility that result to death of plants. F and D shows leaf chlorosis of new leaf whole that is dying off. G and H shows new leaf whole emerging with vigor immediately former dying off.

Discussion

This study reports the first successful creation of octoploids plants from selected tetraploid *Pennisetum purpureum* germplasms through in vitro polyploidy induction using colchicine.Two germplasms, *South africa* and *Bana grass*, were used to produce synthetic mutants. The process involved optimizing a tissue culture method where shoot tillers were cultured on Murashige and Skoog (MS) media with varying concentrations of plant growth hormones (Unami et al., 2012). The most effective media for inducing embryogenic calli and subsequent shoot regeneration was MS supplemented with low levels of BAP, IBA, and 2,4-D (Unami et al, 2012). The study also highlighted the challenges of colchicine use, such as toxicity at higher concentrations, which reduced calli survival and resulted in deformed plants. However, a concentration of 0.1% colchicine over two days proved optimal for inducing polyploidy (Mba et al., 2009) The study observed significant morphological and genetic changes in the induced mutants, such as increased stomatal size, decreased stomatal density, and variations in plant height, tillering, and chlorophyll

expression (Queensenberry et al., 2010). These traits are crucial for selecting superior mutants for breeding programs.

In conclusion, this study successfully advanced napier grass breeding by regenerating polyploidy mutant plantlets through chromosome doubling and somatic embryogenesis, with confirmation of polyploidy via chromosome counting, stomata size and number, Genomic DNA and other morphological characteristics. However, flow cytometry is recommended for further ploidy level confirmation. The superior mutant plantlets can be selected and recommended for characterization across representative agro-ecologies for large-scale production and used in *Penniseturm purpureum* breeding program in Kenya and its environs.

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