International Journal of Bio-resource and Stress Management https://pphouse.org/ijbsm.php

Crossref

IJBSM June 2022, 13(6):543-549 Print ISSN 0976-3988 Online ISSN 0976-4038 **Article** AR2818

Research Article *Stress Management*

DOI: HTTPS://DOI.ORG/10.23910/1.2022.2818

Response of Rice Variety Nagina 22 (N22) and its Putative Mutants to Aluminium Toxicity Conditions in North-East India

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ABSTRACT

The study was performed to evaluate the wildtype genotype Nagina 22 (N22) and its mutants generated by ethyl methyl sulphonate (EMS)for aluminium (Al) toxicity tolerance. Based on performance under upland acidic field conditions on the basis of twelve different traits,among several available M_4N22 mutants,four putative mutants N 714, N 721, N 4249 and N 4487 were selected for hydroponics evaluation. The seeds of the four selected lines were subjected to germination and grown in modified Magnacava's broth supplemented with varying doses of $AlCl_3$ under aseptic conditionsand phenotypic variations for traits like root length, shoot length, root biomass and shoot biomass were recorded. Haematoxylin staining of roots were also carried out on the seedlings grown under control as well as Al toxicity conditions which further revealed that putative mutants N 721 and N 4249 were better performers when compared to N22. Additionally, twelve candidate gene based markers targeting four known Al toxicity tolerance genes resulted in detection of gel based polymorphism for markers AR051-2, AR051-3 and OsFRLD4-1. These putative variations need further validation by running the markers on a set of at least 10 individual putative mutants and sequencing. Al toxicity tolerance in rice is a quantitative trait, these identified mutants can be used to obtain better understanding of tolerance mechanisms and then if possibleuse them synergistically to obtain better tolerance.

KEYWORDS: Al toxicity tolerance, *in vitro*, mutants, polymorphism, rice

***Citation* (VANCOUVER):** Longkumer et al., Response of Rice Variety Nagina 22 (N22) and its Putative Mutants to Aluminium Toxicity Conditions in North-East India. *International Journal of Bio-resource and Stress Management,* 2022; 13(6), 543-549. HTTPS://DOI.ORG/10.23910/1.2022.2818.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

RECEIVED on 08th January 2022 RECEIVED in revised form on 28th May 2022 ACCEPTED in final form on 16th June 2022 PUBLISHED on 27th June 2022

1. INTRODUCTION

Aluminium (Al) is the third most abundant element in the earth's crust and it is detrimental for the growth of the primary producers under acidic soil conditions (Ma et al., 2001). Soil acidity is a naturally occurring phenomenon in the tropical and subtropical geographic regions restricting the crop productivity (Kochian et al., 2005; Das et al., 2017). Almost 40% of the earth's total arable land is affected by the problems caused by the acidic soil conditions (Kochian et al., 2015). In acidic soil conditions, aluminium oxyhydroxides serve as the precursor of the phytotoxic aluminium species, $Al(H_2O)_6^{3+}$, known as Al^{3+} (Abate et al., 2013). Al impairs the biological processes within the root tips and lateral roots (Care, 1995). It also makes the DNA double helix more rigid by reducing the rate of DNA replication (Foy et al., 1992; Ryan and Kochian, 1993), which has a negative impact on DNA composition, chromatin structure and template activity (Minocha et al., 1992). Increased Al concentration in the roots leads to inhibition of root elongation (Yang et al., 2008). The root tip regions therefore, are found to be the primary targets of Al ion (Frantzios et al., 2000). Al has the affinity to form strong bonds with oxygen-donor compounds specifically in the apoplasm and symplasm of root cells (Yamamoto et al., 2001), inducing stubby and brittle root morphology which effects nutrient and water acquisition by the plants (Mossor-Pietraszewska et al., 1997). Even at micromolar concentrations, Al^{3+} inhibits root growth in many agriculturally important plant species (Rahman et al., 2018).

Al toxicity also obstructs the shoot growth by causing nutrient deficiencies, drought stress and hormonal abnormalities (Roy et al., 2014). Exposure to toxic levels of Al gives rise todeficiency of essential elements (Huang and Vitorello, 1996). The symptoms of Al toxicity comprises of curling in young leaves, irregularities in stomatal opening, purpling of stems, retardation in photosynthetic activity, chlorosis and foliar necrosis (Bhalerao and Prabhu, 2013). The biological activity in the roots exhibit more retardation as compared to the shoots (Meriga et al., 2010). Though, amelioration of acidic soils are done through regular application of lime, this practiceis not as feasible since amelioration of sub-soil acidity is a slow and expensive process (Pereira et al., 2010).

Many different mechanisms of Al^{3+} toxicity have been proposed till date which includes cell wall modification, disruption of the plasma membrane and transport processes, interruption of signalling pathways and Al^{3+} binding to the DNA (Brunner and Sperisen, 2013). The term "exclusion" refers to a mechanism that obstructs Al^{3+} from entering the plant (Delhaize et al., 1993), while Al "tolerance" refers to an internal mechanism that detoxifies the Al after Al ions have entered the plant (Li et al., 2014). The most compelling evidence of resistance is based on chelation and exclusion of extracellular Al via Al-activated root organic acid release (Kidd et al., 2001; Tahara et al., 2014).Amongcereals, rice exhibits the highest level of Al tolerance (Ma et al., 2001). Multidrug and toxic compound extrusion (*MATEs*) were first recognised as Al resistance genes using map based cloning of the major Al resistance loci in sorghum and barley (Magalhaes et al., 2007; Furukawa et al., 2007). *MATE* homologues in different crops have been proven tobe responsible for citrate transporteras well as provide Al resistance in the root (Yokosho et al., 2011).

2. MATERIALS AND METHODS

2.1. Experimental site and location

The present study was conducted on a farm located at College of Post Graduate Studies in Agricultural Sciences, Central Agricultural University (Imphal), Umiam, Meghalaya, India during 2015. It is locatedat 25.41° N latitude, 91.54°E longitude and an altitude of 980 m above mean sea level.

2.2. Plant materials and growth conditions

The experimental materials comprised of a wildtype genotype Nagina 22 (N22) and EMS generated four mutant genotypes (M_4 generation) viz. N 714, N 721, N 4249 and N 4487. The mutants were selected based on field performance of M_3 generation. Around 100 seeds of M_3 generation were grown with a row -row and plant-plant spacing of 10 cm. Phenotypic traits such as plant height, number of tillers, number of panicle $hill^{-1}$, panicle length, length of inflorescence, number of spikelet, number of seeds per panicle, % spikelet fertility, flag leaf area, days to 50% flowering, fresh weight and dry weight.

2.3. In vitro experiment

For all *in vitro* experiments, 30-40 seeds from each of the rice genotypes were surface sterilized with 0.1% NaOCl and washed with distilled water. The seeds were then transferred to petri plates containing moist filter paper and kept under controlled conditions [25°C and relative humidity (RH) of 80%] in a plant growth chamber. After germination, the seedlings were transferred to plastic cups containing liquid modified Magnacava's solution. The cups were fixed with sponges punctured with holes and a mesh at the bottom for the seeds to sit on.

2.4. Al toxicity tolerance screening

Al toxicity tolerance screening was performed using modified Magnacava's solution as previously reported by Famoso et al., 2010. The five rice genotypes were screened under hydroponics in both control (without $AlCl_3$) and stress ($AlCl_3$) conditions. For Al toxicity screening for

rice, the germinated seedlings were transferred to plastic cups containing modified Magnacava's solution along with different $AlCl_3$ doses (0, 50, 100, 150 and 200 µM) and grown in aseptic conditions in culture room. After standardization of $AlCl_3$ concentration and duration for evaluating their growth and phenotypic data such asroot length, shoot length, number of lateral roots, root biomass andshoot biomass were recorded on a set of at least 5-10 seedlings grown in both control and stressed conditions. This experiment was repeated three times and the average for each of the parameters was calculated.

2.5. Statistical analysis

Hematoxylin stain was prepared previous to the day of use by combining 0.2% hematoxylin (Merck) and 0.02% potassium iodide and kept overnight. The roots of the seedlings grown in the nutrient media were harvested and soaked in hematoxylin stain for 30 minutes and then washed with distilled water to remove the excess stain. The whole root apices were excised and photographed using a Canon 500D camera with macrolens 90 mmf/2.8. Freehand transverse sections of the roots were cut from the control and treated plants and photographed using a LEICA DM 750 microscope connected to a computer installed with software application suite version 1.8.0.

2.6. Statistical analysis

The data regarding phenotypic parameters for both *field* and *in vitro* experiments were recorded and average of the data (mean), standard deviation (S.D.) and standard error of mean (m±SE) were calculated using Microsoft excel.

2.7. Molecular analysis

DNA was extracted following the sodium dodecyl sulphate (SDS) method of extraction. Briefly, liquid nitrogen was used to ground the frozen leaves using a mortar and pestle. The powdered tissue was then transferred into 2 ml micro-centrifuge tubes. 1 mL of extraction buffer (pre heated to 65°C) was added to the tubes and after securely capping, mixed by gently rotating/shaking. The tubes were then incubated in a water bath at 65°C for 15-20 minutes. The tubes were then allowed to cool for a couple of minutes to relieve the pressure. 700 µL of chloroform: isoamyl alcohol (24:1) was added to each tube and shaken for 5-10 minutes at room temperature. The tubes were then centrifuged at 11,000 rpm for 10-15 minutes. The upper phase was pipetted out to a fresh 1.5 µL micro-centrifuge tube. Further, 2/3 volume of cold isopropanol was added and the tube was mixed thoroughly by inverting after which DNA was allowed to precipitate for 15–20 minutes. The samples were then centrifuged for 15 minutes at 15,000 rpm or higher time at lower speed to get a DNA pellet. The pellet was washed with 70% cold ethanol (500 µl) and subsequent to drying, 50 µl of TE buffer was added and DNA samples stored at 4°C. After the DNA was extracted, polymerase chain reaction (PCR) was carried out using gene based primers targeting genes such as Nramp, glycine rich protein, isocitrate lyase, citrate transporter andmalate transporter. Actin gene was used as housekeeping gene. For a total of 33 cycles,each PCR cycle run in a thermal cycler consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes for a total of 33 cycles. PCR products were subjected to electrophoresis in 0.8% agarose gel stained with ethidium bromide at 80 V for 2 hours in 0.5XTBE buffer. The agarose gels were visualised using a gel documentation system (AlphaImager) connected to a computer.

3. RESULTS AND DISCUSSION

3.1. Screening under field conditions

It is known that Al toxicity conditions affect crop productivity (Kochian et al., 2005) by affecting root tips and lateral roots (Care, 1995). Mutants in rice (Huang et al., 2009) and Arabidopsis (Zhu et al., 2012) have been identified for Al toxicity and studied with respect to root traits and affect of cell wall, respectively. The four putative mutant genotypes namely, N 714, N 721, N 4249 and N 4487 were screened along with the parent line N22 for Al toxicity tolerance under field condition (Table 1 and 2). N 4249 recorded the highest mean value for plant height (123.46±0.79 cm) followed by N 4487 (107.5±9.67 cm), N 721 (81.5±0.87 cm) and N 714 (79.66±3.94 cm). In the case of inflorescence length, N 4249 recorded the highest mean value (22.83±0.6 cm) followed by N 714 (19.5±1 cm) and N 4487 (19±2.47 cm) while N 721 recorded the least mean value (18.66±0.33 cm). For panicle length, N 4249 recorded the highest value of 56.66±2.03 cm followed by N 4487 (50.13±7.12 cm) and N 714 (47.5±2.25 cm) while N 721 recorded the least mean value (40±1.73 cm) for this trait. The selected plants showed similar values for the total number of leaves at an average value of 4 for all the other three genotypes except for N 4249 which recorded a reading of 5 leaves $plant^{-1}$.

For the tillers number per plant, N 721 recorded the highest value of 12 followed by N 714 (9) and N 4249 (9) while N 4487 recorded the least mean value for the particular trait (6).For total number of panicles, N 721 recorded the highest mean value (11±0.58) followed by N 714 (10±0.67) and N 4249 (9±2.52) while N 4487 recorded the least mean value (6±1.53).

In the case of number of spikelets per panicle, N 714 recorded the highest mean value (10±0.67) followed by N 4249 (8±0.58) and N 4487 (6±1) while N 721 recorded the least mean value (4±0.33). Genotype N 4249 recorded

Table 1: Phenotypic evaluation between wild type parent N22 and four putative M5 generation rice mutant lines (mean±SD) for different yield parameters under upland acidic field conditions

Geno-type	Plant height (cm)	Panicle length (cm)	No. of leaves	No. of tillers hill^{-1}	Filled grains panicle^{-1}	% spikelet fertility	Flag leaf area (cm^2)	Fresh weight (g)	Dry weight (g)
N 22	92.66±4.34	42.66±2.4	4	15	29±15.24	6.58±2.05	29.39±2.15	54.13±13.79	23.50±3.36
N 714	79.66±3.94	47.5±2.25	4	9	430±110.15	36.11±15.23	25.39±2.19	48.93±8.14	21.61±1.24
N 721	81.5±0.87	40±1.73	4	12	144±10.21	37.13±10.37	25.88±4.06	77.83±7.53	29.5±2.58
N 4249	123.46±0.79	56.66±2.03	5	9	260±109.27	31.76±9.34	32.93±0.97	63.65±16.54	25.61±4.08
N 4487	107.5±9.67	50.13±7.12	4	6	120±64.09	39.22±16.23	33.18±11.38	30.76±7.53	15.4±4.22

Table 2: Evaluation of different phenotypic parameters (n=15±SD) of four putative mutant M4 rice genotypes along with the parent N22 for Al toxicity tolerance using Magnacava nutrient media at pH 4.2

Genotype	Shoot length control (cm)	Shoot length treated (cm)	Root length control (cm)	Root length treated (cm)	Shoot biomass control (g)	Shoot biomass treated (g)	Root biomass control (g)	Root biomass treated (g)
N 22	21.54±0.30	14.17±0.40	8.74±0.30	6.61±0.15	0.19±0.004	0.12±0.003	0.12±0.008	0.07±0.002
N 714	17.53±0.32	14.21±0.22	9.36±0.25	6.21±0.15	0.14±0.006	0.11±0.002	0.15±0.008	0.07±0.001
N 721	19.41±0.39	16.04±0.33	8.26±0.25	6.54±0.18	0.16±0.006	0.12±0.002	0.11±0.006	0.07±0.002
N 4249	18.09±0.41	14.01±0.40	8.02±0.19	6.00±0.12	0.14±0.004	0.10±0.003	0.09±0.003	0.07±0.001
N 4487	21.31±0.46	14.69±0.23	7.85±0.23	5.47±0.17	0.17±0.005	0.11±0.002	0.09±0.004	0.06±0.002

the highest mean value (11±0.37) for number of seeds/panicle followed by N 714 (9±0.06) while N 4487 and N 721 recorded the least mean value for the particular trait (8±0.59) and (8±0.68), respectively.

In the case of % fertility, N 4487 recorded the highest mean value (39.22±16.23%) followed by N 721 (37.13±10.37%) and N 714 (36.11±15.23%) while N 4487 recorded the least mean value for the particular trait at 31.76±9.34%. Genotype N 4487 recorded the highest mean value (33.18±11.38 cm^2) for flag leaf area followed by N 4249 (32.93±0.97 cm^2) and N 721(25.88±4.06 cm^2) while N 714 recorded the least mean value for the particular trait (25.39±2.19 cm^2).

In the case of fresh weight, N 721 recorded the highest mean value (77.83±7.53 g) followed by N 4249 (63.65±16.54 g) and N 714 (48.93±8.14 g) while N 4487 recorded the least mean value for the particular trait (30.76±7.53 g).

In the case of dry weight, N 721 recorded the highest mean value (29.5±2.58 g) followed by N 4249 (25.61±4.08 g) and N 714 (21.61±1.24 g) while N 4487 recorded the least mean value for the particular trait (15.4±4.22 g).

Previously, genotypic variation for Al toxicity tolerance has been reported in various species like rice (Famoso et al., 2010), barley (Furukawa et al., 2007), soyabean (Foy et al., 1992), etc to name a few. Both root (Kidd et al., 2001; Tahara et al., 2014) and shoot (Bhalerao and Prabhu, 2013) traits have been reported to show variation under Al toxic conditions.

3.2. Screening under in vitro conditions

Apart from screening in field conditions, the selected putative mutants were also screened for seven days under *in vitro* conditions for seedling stage Al toxicity tolerance (Figure 1). In the case of rice, modified Magnacava's solution as previously reported by Famoso et al., 2010 was used for Al toxicity tolerance screening. Rice is the most tolerant to Al toxicity among cereals (Ma et al., 2001), and genetic background also plays a vital role in toxicity tolerance (Famoso et al., 2010). Therefore, an attempt was made to understand Al toxicity tolerance mechanism using N 22 mutants.

In Al toxicity conditions, while the parent genotype N 22 recorded better mean value at 6.61±0.15 cm, among the putative mutant genotypes, N 721 recorded the best mean value (6.54±0.15 cm) followed by N 714 (6.21±0.15 cm) and N 4249 (6±0.12 cm). N 4487 recorded the least mean value at 5.47±0.17 cm. while in control condition, N 714 recorded the highest mean value reading at 9.36±0.25 cm followed by N 721 (8.26±0.25 cm) and N 4249 (8.02±0.19 cm). N 4487 recorded the least reading at 7.85±0.23 cm. The root length

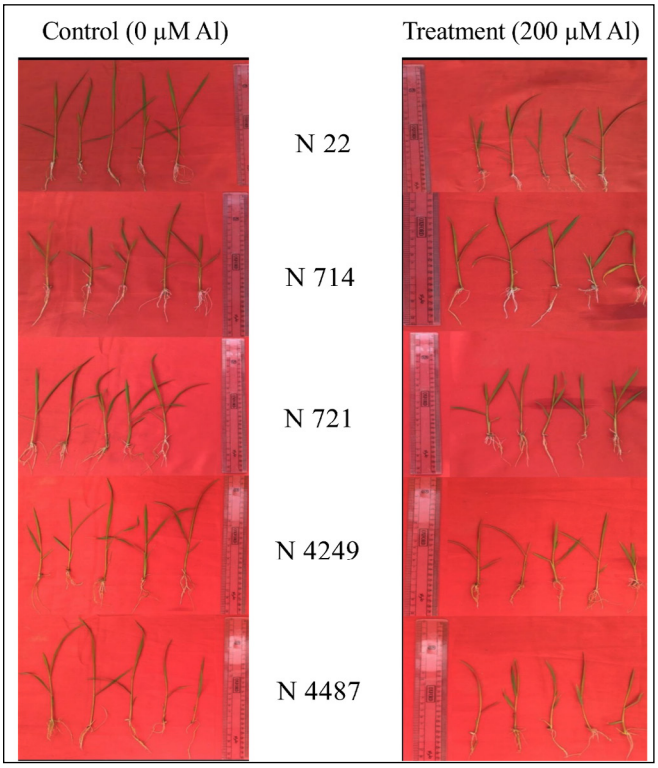

Figure 1: A comparison of phenotypic differences among different rice genotypes (parent-N 22; M5 putative mutants) under Al toxicity hydroponic conditions (Control- 0 µM Al; Treatment- 200 µM Al)

of the parent was found to be 8.74±0.30 cm. All the other parameters like shoot length, root and shoot biomass were significantly less in Al toxicity conditions when compared with no Al condition (control) for all the five genotypes. The difference between the control and treatment were statistically significant.

In the case of shoot length in treatment conditions, N 721 recorded the best mean value (16.04±0.33 cm) among the putative mutant genotypes, followed by N 4487 (14.69±0.23 cm) and N 714 (14.21±0.22 cm). N 4249 recorded the least mean value at 14.01±0.40 cm while in controlled solution, N 4487 recorded highest mean value reading at 21.31±21.31 cm followed by N 721 (19.41±0.39 cm) and N 4249 (18.09±0.41 cm). N 714 recorded least reading at 17.53±0.32 cm. The shoot length of the parent was found to be 14.17±0.40 cm in treated solution while in control solution the shoot data was found to be 21.54±0.30 cm.

The root biomass in treated solution in putative mutant genotypes N 714, N 721 and N 4249 was 0.07±0.001 g, 0.07±0.002 g and 0.07±0.001 g, respectively. While for the parent N 22 it was 0.07±0.002 g. N 4487 recorded the least mean value at 0.06±0.002 g. In absence of Al, N 714 recorded the highest mean value reading at 0.15±0.008 g followed by N 721 (0.11±0.006 g). N 4249 and N 4487 recorded the least readings at 0.09±0.003 g and 0.09±0.004 g, respectively.

In the case of shoot biomass in treated solution, the parent genotype N 22 recorded better mean value at 0.12±0.003 g, while N 721 recorded the best mean value (0.12±0.002 g) among the putative mutants, followed by N 714 (0.11±0.002 g) and N 4487 (0.11±0.002 g). N 4249 recorded the least mean value at 0.10±0.003 g.

3.3. Hematoxylin staining and sectioning

Transverse sections of the roots were prepared from the selected four putative mutants and parent rice genotypes after treating the roots of the selected individuals with hematoxylin stain. The sectioned roots were then viewed under LEICA DM 750 microscope to visualisethe intensity and extent of staining, if any in the roots. The pictures of the sectioned roots were taken at the magnification of 10X and 40X. The roots sections of both control and treated roots were scored based on the intensity of the stain (Figure 3). Previously, it has been shown that aluminium localisation can be detected by using staining method and it is correlated with tioxicity symptoms in roots (Alvim et al., 2012).

From the microscopic sections, it was observed that the root sections under control conditions did not take up the hematoxylin stain at all. On the other hand, sections from roots exposed to Al had varying intensity of the staining. The treated roots of M 27, N 721 and N 4249 took up less

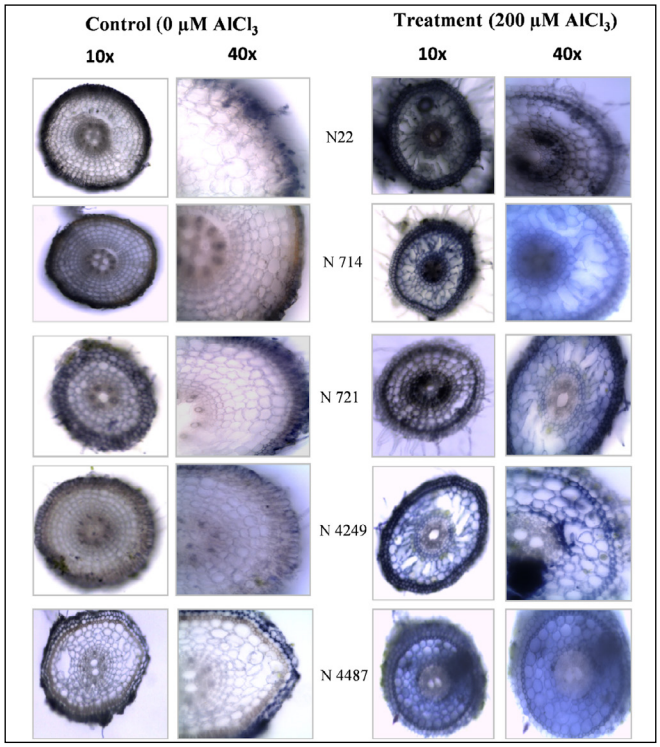

Figure 2: Comparison of hematoxylin stained and sectioned roots of N22 and four M5 putative mutants grown under in vitro conditions at pH 4.2. The roots under stressed conditions take up more stain as compared to the roots under control.

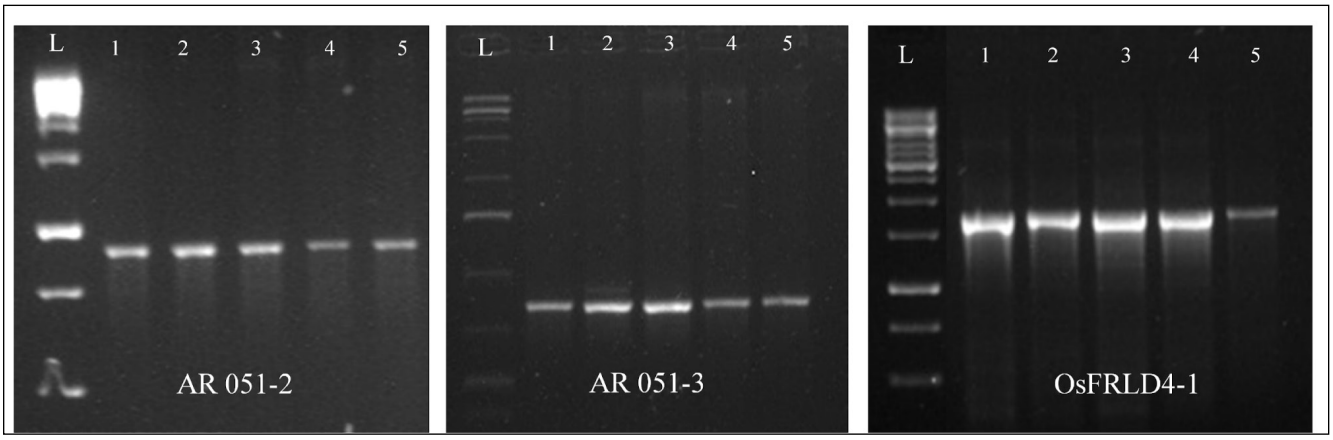

Figure 3: Agarose gel pictures showing banding pattern targeting four genes in rice genotype N22 and its four putative M5 mutants. The name of the primer is indicated at the bottom of each gel, respectively. L=ladder, 1= N22, 2=N 714, 3= N 721, 4= N 4249 and 5= N 4487

stain. The intensity of hematoxylin stain was visibly higher in N 714 and N 4487 (Figure 2).

3.4. PCR analysis

DNA extracted from the parent N22 and its four putative M_5 mutants were used for PCR analysis using a set of 12 markers targeting four genes for Al toxicity tolerance. Polymorphism was observed only for the markers AR051-2, AR051-3 and OsFRLD4-1 which are targeting genes responsible for glycine rich protein (AR051-2, AR051-3) and citrate transporter (OsFRLD4), respectively (Figure 3). This suggests that these genes and markers could be utilised effectively for better response to Al toxicity conditions in rice. Previously, citrate transporters have been reported to provide Al resistance (Yokosho et al., 2011).

4. CONCLUSION

Malate-specific transporter, citrate-specific transporter and Al-specific transporter along with glycine-rich protein and isocitrate lyase were targeted to understand the molecular basis of tolerance in rice. Gel based polymorphism was obtained for three markers namely, AR051-2, AR051-3 and OsFRLD4-1 which underlie a glycine rich protein (AR051-2, AR051-3) and citrate transporter (OsFRLD4), respectively. Further validation needs to be carried out to ascertain the level of tolerance due to the mutations so observed.

5. ACKNOWLEDGMENT

This work was supported by funds from Indian Council of Agricultural Research (ICAR) through National Agricultural Innovative Project (C30033/415101-036) and Central Agricultural University, Imphal CAU (I). The seeds for N22 wildtype and mutants were provided by Dr. N Sarla (ICAR National Professor, Indian Institute of Rice Research, Hyderabad).

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