Tree Leaf Extracts as Biostimulants for influencing Germination Attributes and Biochemical Changes in kodo millet (*Paspalum scrobiculatum* L.)

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Abstract

This study was performed at the Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625104, India with a primary objective of evaluating the effectiveness of natural botanicals/tree leaf extracts of three horticultural tree species on germination and associated features in kodo millet and, in coordination with bioassay investigations, biochemical analysis of leaf extracts and enzyme activities in kodo millet. Response Index was computed for comparison and the reasons for the effect, if any, was investigated by GCMS-based identification of the chemicals found in the leaf extracts. The findings showed that treating the seeds with mango leaf extract boosted the seedling vigour index in kodo millet. In addition, the higher response on germination and enzyme activities such as dehdrogenase, α Amylase, catalyse and peroxidase in kodo millet were documented in Moringa oleifera leaf extract.

Key words: *Bioassay, Mango leaf extract, Moringa oleifera, Germination, kodo millet*

1. Introduction

Agriculture is faced with the challenge of developing more efficient and sustainable alternatives to expedite the reduction of malnutrition, poverty, starvation, and energy and water usage while parallelly enhancing the productivity and the quality of crops due to the exponentially growing population, limited available farmland, genetic potential of crops, depletion of natural resources, and climate change ([1]; [2]; [3]). Food security for the world's expanding population is a major concern for governments and policy -makers. Millets have recently gained recognition as important substitutes for staple grains in an effort to address global food shortages and meet demand from both emerging and industrialised countries due to population growth [4]. The kodo millet is scientifically called as *Paspalum scrobiculatum*. L and believed to have originated in India. It is thought that the domestication of Araka or kodo millet occurred around 3000 years ago [5]. It seems to only be produced in large regions of Karnataka, Gujarat, Chhattisgarh, Eastern Madhya Pradesh and parts of Tamil Nadu in India. Kodo grains have been shown to be an excellent starving reserve with 8.35 per cent protein, 1.45 per cent fat, 65.65 per cent carbohydrate, and 2.95 per cent ash. It is renowned for having the strongest drought resistance crop among the small millets and expected to produce good yield in a short amount of time [6].

Demands for high-quality foods are increasing along with the world's population, which forces farmers to develop novel non-chemical solutions to increase food supply and quality [7]. Alternatives to synthetic agents to enhance crop growth and development have a great deal of potential owing to the global rise of organic farming. One such substitute that can positively influence growth, seed germination, foliar nutrition, tolerance to abiotic stresses, and post-harvest quality is the use of natural biostimulants (substances originating from plants) ([8] and [9]). Biostimulants that can accelerate plant development [10], improve a plant ability to respond to stress, and improve physiological processes in plants [11]. The regards to germination, seed priming, a presoaking method that accelerates the physiological process of seed germination has been used in the past [12]. The productivity and quality of crops can be enhanced because to the abundance of nutrients and beneficial compounds found in plant byproducts ([13] and [14]). Moringa ([15]; [16] and [17]) and *Annona squamosa* leaf extracts [18] are more renowned as biostimulants because they are more potent than synthetic growth promoters and since they are less costly and include minerals, antioxidants, and hormones including auxins, gibberellins, and cytokinin. Alkaloid [19], flavonoid [20], triterpenoid and saponin are some of the groups of secondary metabolite compounds that have been extracted from plants and exploited as biostimulants [21].

Consequently, the following objectives were established for this study: In order to. i) Test the effectiveness of botanicals as biostimulants and growth promoters in kodo millet ii) to identify the positive or inhibitory effects of selected tree leaf extracts on kodo millet seed germination.

2. Materials and methods

The laboratory experiments were performed in seed lab for germination related works and biotechnology lab for GCMS studies at Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625104, Tamil Nadu, India. For botanical extracts preparation, fresh and clean leaves of three horticultural tree species viz., *Mangifera indica*, *Moringa oleifera* and *Annona squamosa* from the campus orchard were collected, ground with distilled water at a 1:1 ratio, and extracts were filtered and kept as stock solution. From the stock solution, 5 percent solution was prepared and kodo millet (Co 3) seeds were soaked in leaf extracts for 12 hours [43].

2.1. Laboratory experiment setup

[22] recommended using the roll-towel method, which entails spreading seeds out on germination paper and rolling them, to assess germination and related attributes in the initial laboratory experiment. On germination paper, 25 numbers of kodo millet seeds were placed and germination papers were placed vertically in sterilised conical flasks, one-fourth filled with the three leaf aqueous extracts [23]. To evaluate the rate of germination, a different germination test was performed simultaneously in petri plates, where seeds were placed in round germination paper. For both tests, distilled water served as control. Three replications have been used in the experiments that were set up in CRD. Up to 14 days, germination and related traits in treated and control seedlings were studied. At the conclusion of the research, overall seedling length and daily germination counts were recorded. A daily count of germinates has been used to construct a set of germination indices, as shown below.

2.2. Measurement and analysis of data

2.2.1. Germination Energy: [24] proposed the germination energy (GE) formula as

$$GE = \frac{X1}{Y1} + \frac{X2-X1}{Y2} + \ldots\ldots\ldots\ldots + \frac{Xn-Xn-1}{Yn}$$

Where X_n = number of germinant on n^{th} counting date; Y_n = number of days from sowing to n^{th} count.

2.2.2. Germination value: The germination value provided by [25] was

$$GV = (\sum DGS / N) \times \frac{GP}{10}$$

Where GV = germination value, GP = Germination per cent at end of test, DGS = Daily germination speed obtained by dividing cumulative germination percent by number of days since sowing, $\sum$ DGS = Summation of all DGS figures, N= Number of daily counts effective from date of first germination.

2.2.3. Emergence Energy Value: EEV is the highest value obtained by dividing the germination percentage on a given day by the number of days since the test started, when that germination percentage was attained [26].

2.2.4. Germination Relative Index: (GRI) was calculated using the formula suggested by [27].

$$GRI = [\sum Xn\ (h - n)]$$

Where, Xn = number of germinant at n^{th} count, h = total number of counts and n = count number.

2.2.5. Response index: Response index [28], which was computed as follows, was used in bioassays to assess the degree of inhibition vs. simulation

if T>C, RI=1-(C/T)

if T = C, then RI = 0

if T<C, then RI= (T/C) -1

where, T is treatment mean and C is control mean. A negative RI reflects proportional disparity in output (germination, radicle length or plumule growth) of test crop in treatment relative to output in control.

2.3. Biochemical analysis

2.3.1. Dehydrogenase activity (OD value)

The dehydrogenase activity of seeds was estimated by following the method of [29] with minor modifications. Twenty five seeds from each treatment were preconditioned for 12 h in between folds of moistened filter papers and five embryonic axes were excised and incubated in darkness with 5 ml of 0.5% tetrazolium chloride solution in glass vials for three hours. After incubation (3 h), the tetrazolium chloride solution was decanted and the embryos were thoroughly washed with distilled water and surface dried with blotters. The formazan was eluted by soaking the stained embryos in 5 ml of methyl cellosolve (2 methoxyethonal) for 1h and the optical density was measured using Cary UV spectrophotometer at 480 nm.

2.3.2. α- amylase activity (units/gram)

Two replicates of 500 mg of pre-germinated seed samples were homogenized in 1.8 ml of cold 0.02 M Sodium phosphate buffer (pH 6.0) and centrifuged at 20,000 rpm for 20 minutes to extract enzymes. To 0.1 ml of enzyme extract, one ml of 0.067 per cent starch solution was added. The reaction was stopped after 10 minutes of incubation at 25^{o}C by the addition of one ml of Iodine HCLL solution (60 mg KI and 6mg I_2 in 100 ml of 0.05 N HCl). Change in colour was measured at 620 nm. The activity was calculated using the following formula and expressed as mg maltose. min^{-1} [30].

$$\text{α- amylase activity} = \frac{\text{OD value}}{\text{Volume of sample pipetted out}} \times \frac{1000}{250}$$

2.3.3. Catalase activity

The catalase activity of seeds was estimated in duplicate following the method of [31], with modifications. For assessing catalase activity, seeds were pre conditioned. One gram of embryos were ground in a pestle and mortar with 20 ml of 0.067 M phosphate buffer by dissolving 3.522 g of $KH_2\ PO_4$ and 7.268 g of $Na_2HPO_4\ 2H_2O$ in distilled water and the volume was made up to one litre (Assay buffer diluted 10 times) at 4^0C and centrifuged at

15000 rpm for five min. The supernatants were used for enzyme assay. In experimental cuvette, three ml of H_2O_2 phosphate buffer (0.16 ml of H_2O_2 (10% w/v) diluted to 100 ml with phosphate buffer – prepared fresh) and 0.02 ml of sample (1 ml of sample diluted to 10 ml) were added and mixed well with a glass rod. The time (Δt) required for decrease in absorbance was noted at 240 nm in Cary UV spectrophotometer. Catalase activity was expressed as units/g tissue using the following formula.

$$\text{Catalase activity (units/g)} = \frac{17 \times 10 \times 20 \times 1000}{\Delta t \times X \times Y}$$

Where,

Δt – Time required to decrease the absorbance
X – Volume of enzyme extract
Y – Volume of buffer solution

2.3.4. Peroxidase activity

The peroxidase activity of seeds was assessed in duplicate following the method of [32], with modifications. Kodo millet seeds were preconditioned as described elsewhere. One gram of embryos was homogenized in pestle and mortar with three ml of 0.1 M phosphate buffer (pH 7). The homogenate was centrifuged at 10000 rpm for 10 min. The supernatant was the enzyme source. To three ml of H_2O_2 (0.142 ml of H_2O_2 diluted to 100 ml), 0.1 ml of enzyme extract (sample) was added. The time (Δt) required for increase in absorbance in UV spectrophotometer at 436 nm was noted. All procedures were carried out at 5^0C. Peroxidase activity was expressed as units / g of sample.

$$\text{Peroxidase activity (units/g)} = \frac{3.18 \times 0.1 \times 1000}{39 \times W \times \Delta t \times X \times 1000}$$

Where Δt – Time required increasing the absorbance
X – Volume of enzyme extract
W – Weight of the sample

3. Results and discussion

3.1. Influence on germination, shoot length, root length, and vigour index

Investigation has examined the effect of three leaf extracts on kodo millet germination and related variables were presented in Table 1. Kodo millet germination was found to be highest in moringa leaf extract, which is 7% greater than the control. Higher germination percentage was a critical factor in determining seed volume and viability [33], which is aided by the presence of active ingredients [34], micronutrients [35], and biologically active compounds such as phenolic compounds, organic acids, proteins, and alkaloids [36] in natural plant leaf extracts. However, Moringa extracts produced the longest root of 9.21 cm whereas mango leaf extract had longest shoot (8.42 cm) in kodo millet as evidenced from

findings of [37] and [38]. Additionally, according to [44], the use of 2% leaf extract and 3% branch extract of *M. oleifera* the two times (7 and 14 days later in a planting season, notably (planting) recorded higher plant, fresh and dry weight of sativa subspecies of *Eruca vesicaria*. This result is in line with [46, 47, 48, 49, 50] in sorghum, tomato, coriander, wheat and pea. Similarly, mango leaf extract showed a maximum seedling dry weight of 19 mg in kodo millet.

Table 1. The influence of tree leaf extracts on germination and associated parameters in kodo millet (*Paspalum scrobiculatum* L.)

	Germination %	Root length (cm)	Shoot length (cm)	Seedling dry weight (mg)
Moringa leaf extract @ 5%	92.00 ± 1.298^{a}	9.21 ± 0.139^{a}	7.75 ± 0.081^{b}	13.00 ± 0.026^{b}
Mango leaf extract @ 5%	88.00 ± 0.486^{ab}	8.08 ± 0.082^{b}	8.42 ± 0.157^{a}	19.00 ± 0.032^{a}
Annona leaf extract @ 5%	90.00 ± 1.453^{a}	7.52 ± 0.125^{c}	7.07 ± 0.072^{c}	13.00 ± 0.155^{b}
Control	86.00 ± 0.731^{b}	7.73 ± 0.026^{bc}	6.46 ± 0.060^{d}	13.00 ± 0.271^{b}
SE. d	1.7333	0.1640	0.1697	0.2725
C.D. (0.01)	5.8161	0.5503	0.5695	0.9142

*Data are the mean values of replicates with ± standard error.

3.2. Effect of tree leaf extract on the Response Index in kodo millet

Influence on individual tree leaf extracts on seed germination and seedling growth in kodo millet, resulting in a wide range of response index values when compared to control (Table 2.). The response on germination and root length being obtained from Moringa leaf extract, whereas the highest and positive RI in seedling dry weight, seedling vigour index I and II were acquired in mango tree leaf extract when compared to moringa and Annona. All three leaf extracts showed predominantly positive RI values in the current investigation, indicating that they are all-natural growth biostimulants. In the present investigation, all three leaf extracts had predominantly positive RI values, proving that they are all-natural growth biostimulants. This finding was supported by [39] in *G. linearis* leaf extract.

Table 2. Response Index of tree leaf extracts on kodo millet (*Paspalum scrobiculatum* L.) seed germination and related attributes

Treatments	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling dry weight (mg)	SVI I	SVI II
Moringa	+0.065	+0.161	+0.166	0.000	+0.218	+0.065
Mango	+0.023	+0.043	+0.233	+0.316	+0.160	+0.331
Annona	+0.044	-0.027	+0.086	0.000	+0.071	+0.044

3.3. Performance of tree leaf extract on germination related attributes

Influence of tree leaf extracts on germination attributes was presented in Table 3. Germination energy measures the fraction of seeds that germinate rapidly and has an influence on the viability and vigour of seedlings [22]. The highest germination energy value (5.31), as well as the required germination values (21.08) and germination relative index (473.5), were all obtained by the moringa leaf extract, whereas the highest emergent energy value (4.40) was attained in mango leaf extract. That might be due to tree leaf extracts contain diverse of phytochemicals, such as phytosterols, glycosides, essential oils, saponins, phenols, and flavonoids, which act as a precursor to GA_3 due to saponins and presence of terpenoids, phytosteroids, fatty acids, and glycosides ([40] and [41]).

Table 3. Influence of various tree leaf extracts on kodo millet (*Paspalum scrobiculatum* L.) germination-related characteristics

Treatments	Germination energy	Germination Value	Emergence Energy value	Germination Relative Index
Moringa	5.31	21.08	3.83	473.5
Mango	5.26	20.07	4.40	456.5
Annona	5.12	19.82	3.75	461.5
Water	4.94	18.37	3.58	442.5

3.4. Potential effect of tree leaf extract on biochemical alterations in kodo millet seeds

This study discovered that seed fortification with botanicals/leaf extracts increased enzyme activity of EC, dehydrogenase, catalase, peroxidase, and amylase (Fig. 1 & Fig. 2). Fortification reduces sugars while increasing amylase activity, allowing reserves to be mobilised from seed store to embryo, resulting in enhanced early seed vigour. This has been revealed in current experiment as an application of 5% moringa leaf extract recorded higher amylase, dehydrogenase and peroxidase activities in kodo millet. According to [42, 45] this might be due to the presence of compounds that promote growth may increase respiration rates and antioxidant enzymes contribute to crop development. The chromatogram of mango leaf exhibited the peak compound area of Catechol (15.50 %), Cyclohexanone, 2-pentyl- (15.05%), 2-Dodecylcyclobutanone (11.84%) with the retention time of 13.241, 16.986 and 17.527, respectively.

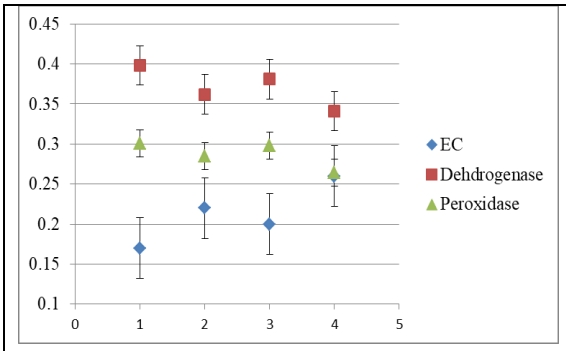

Fig. 1. Influnce on botanicals on EC, Dehydrogenase and peroxidase enzyme activities

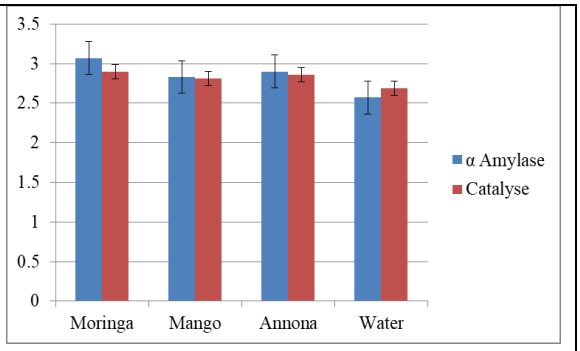

Fig. 2. Influnce on botanicals on α amylase, and catayse enzyme activities

3.5. Correlation matrix on germination and related attributes

Table 4. illustrated that, germination had positive correlation on root length, shoot length and seedling vigour index is (r = 0.67**, 0.38** and 0.76**) while negative impact was observed in seedling dry weight and seedling vigour index II (-0.26** and -0.13**). However, root length between other germination attributes indicated the positive correlation except seedling dry weight while shoot length exhibited positive correlation regarding with all germination attributes. The positive correlation indicated that application of natural tree leaf extract exhibited the positive results in kodo millet seed germination and its related attributes.

Table 4. Studies on correlations between the effects of various leaf extracts on kodo millet (*Paspalum scrobiculatum* L.)

Attributes	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling dry weight (mg)	Seedling vigour index I	Seedling Vigour Index II
Germination (%)	1					
Root length (cm)	0.67**	1				
Shoot length (cm)	0.38**	0.47**	1			
Seedling dry weight (mg)	-0.26**	-0.05**	0.78**	1		
Seedling Vigour Index I	0.76**	0.87**	0.81**	0.29**	1	
Seedling Vigour Index II	-0.13**	0.04**	0.85**	0.99**	0.40**	1

** Indicates significant at 1% level of probability

4. Conclusion

Nowadays orchards have been established with an objective of enhancing fruits production to meet human needs. Apart from producing the economic part, fruits, those trees are also capable of accumulating secondary metabolites in their leaves and other parts. Such secondary metabolites can be exploited for various applications in crop production, based on their natural characteristics. This study made an attempt on this line and brought forth some good results on utilizing the extracts of tree leaf as biostimulant. Furthermore, this has also illuminated a unique area of research on sustainable biological agriculture known as positive allelopathy.

Fortifying the seeds of kodo millet with *Moringa oleifera* @ 5 percent leaf extract had improved germination and root length, whereas fortifying seeds with *Mangifera indica* @ 5 percent leaf extract had improved seedling vigour index I and II. Nevertheless, all three leaf extracts showed a positive response index (RI) on germination-related parameters and biochemical alterations in kodo millet seeds. To conclude, the findings scientifically validated the use of tree leaf extracts as a growth bio-stimulant in crop production when compared to synthetic chemical treatments and facilitate to generate chemical-free, high-quality food grains in kodo millet.

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