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Analysis of Intraspecific Diversity in Two Green Capsuled Accessions of *Bixa orellana* L., a Food-Dye Plant by Single Sequence Repeat (SSR) Markers

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Abstract: ***Bixa orellana*** **L., is a natural food dye yielding plant belonging to the family Bixaceae. It yields a non-toxic, edible Food dye Bixin and Nor-Bixin also popularly known as Annatto having orange-red colour and has a huge demand in the dairy industry to colour food products. In the present study, the 2 accessions of *Bixa orellana* L with white and rose-coloured flowers both bearing green capsules with green spines in common were identified from west and south of Bangalore city (Bangalore University and Lalbagh), Karnataka, India. These 2 accessions were analyzed for intraspecific variations with respect to the morphology, Genetic relatedness and Dye (Bixin) content. Our studies report that these 2 accessions were highly diverse and exhibited maximum variations with respect to habit and other morphological parameters. Hence these 2 accessions were subjected to SSR (Single Sequence Repeat) Marker studies. The analysis of SSR data showed high level of genetic variations. Out of 10 SSR primers, 7 primers showed polymorphism between the two accessions. A total of 43 discernible and reproducible SSR bands were generated by employing 7 selected primers. Out of them, 22 bands were polymorphic. The number of bands produced per primer ranged from 1-8. The markers described in this study could be of use in marker assisted selection, genetic improvement and breeding programmes and could help in the utilization and germplasm conservation of Rose-coloured flower and Green capsuled with green spined accession of *Bixa orellana* L., in systematic scientific breeding programmes which also yields maximum bixin content.**

Index Terms: ***Bixa orellana*** **L., Bixin, green capsule, green spines, rose colour flower, SSR markers and white colour flower.**

I. INTRODUCTION

Bixa orellana L., a native of tropical America is a food dye and medicinally important plant belonging to the family Bixaceae. It is cultivated in different parts of the world for the food dye, known as annatto extracted from the seeds. Annatto (E- 160b) is one of the 13 basic food pigments approved by US FDA and ranks 2nd in the world. The dairy industry, cosmetic industry and textile industry uses annatto for colouring ice-creams, butter, lipsticks, hair oils and dyeing silk and Cotton clothes respectively (Poornima, 2014).

Bixa orellana L., is a highly cross-pollinated plant which exhibits variability in the saplings obtained from seeds. There is also variation in the composition of the seed bixin content among the trees and this limits marketability (Ambika & Poornima,2004). The earlier studies by (Poornima,2006) and (Poornima et al, 2009 & Poornima & Ambika 2012) had reported the intraspecific diversity among 20 different accessions of *Bixa orellana* L., identified from different parts of Bangalore, Karnataka and reported 5 divergent accessions with regard to morphology with respect to flower and capsule colour and seed bixin content by RAPD analysis.

Adding to the list of above identified 5 divergent accessions, the 6th accession of *Bixa orellana* L., bearing a Rose colour flower and green colour capsule with green spines was reported for the first time from Lalbagh, Bangalore, Karnataka and the morphological studies were undertaken on the same (Poornima, 2014). Since only the morphological data was available as above and no data was available on both the genetic diversity and seed bixin content in the literature on 2 accessions of *Bixa orellana* L., with white flower and rose-coloured flower with green capsule and green spines, a detailed study was undertaken and ours is the first of its kind in this regard to identify the elite variety in terms of flower and capsule colour and total bixin content amongst the same.

Hence our study aimed to explore the existence of intraspecific diversity in the *Bixa orellana* accessions with white

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and rose-coloured flowers bearing green capsules with green spines in common obtained from 2 different areas of Bangalore City (Bangalore University and Lalbagh), Karnataka, India.

II. Materials & Methods

Biological Samples: a) Accession 1: Bangalore University White-coloured flower with green capsule and green spines. b) Accession 2: Lalbagh Rose-coloured flower with green capsule and green spines.

A. Morphological Studies

The present study involves *Bixa orellana* L., ear marked from 2 localities- Bangalore University and Lalbagh, West and South of Bangalore, Karnataka, India. The passport data of these plants were collected. These were the size and the height of the plant, branching pattern, Number of branches, leaf shape, flowering time, flower and stamen colour, fruiting period, capsule shape, size and its texture, seed length, width and number of seeds per capsule.

B. Assessment of Intraspecific Genetic Diversity Using Single Sequence Repeat (SSR) Markers-Plant Genomic DNA isolation and PCR (Polymerase chain Reaction), Amplification and Genotyping of Single Sequence Repeats (SSR).

The Genomic DNA was extracted using fresh young leaves collected from both the accessions of *Bixa orellana* L., using Nucleospin ® tissue kit with slight modifications (Machery & Nagel, 2017). The DNA concentration and purity were determined by using Spectra Max® i3x device and dilutions were made when necessary. Ten SSR markers developed for *Bixa orellana* L. were used in the present study These markers were selected based on their polymorphism and compatibility for multiplexing. (Table I).

The Polymerase Chain Reactions of DNA samples was performed in an applied bio system by Thermo Fisher Scientific veriti 96 well Thermal Cycler for 40cycles (3hrs 24mins). To check the quality of PCR amplifications, they were subjected to electrophoresis on agar rose gels (1.2% (w/v)) stained with Gel Red (Biotium). The PCR products visualized in Gel Doc™ XR+ Gel Documentation System (BIO-RAD). DNA fragment separation and detection were performed using PyElph1.4 software.

C. Estimation of Total Pigment (Bixin) Content

The outer covering of the seed is covered with the pigment, Bixin. The total soluble pigment (Bixin) from the seeds was extracted and estimated (Mc Kneown & Mark 1962).

D. Statistical Analysis

For SSR marker profile, the visible bands of each accession were recorded as binary data: 1= presence of band and 0=absence of bands. The data obtained for total pigmen t(bixin) content from both the accessions of *Bixa orellana* L. were subjected to statistical analysis using T-Test.

III. Results

A. Morphological Studies

Both the accessions of *Bixa orellana* L., collected from 2 different areas of Bangalore (Bangalore University and Lalbagh), Karnataka, showed wide morphological variations with respect to the size and the height of the plant, branching pattern, Number of branches, leaf shape, flowering time, flower and stamen colour,

fruiting period, capsule shape, size and its texture, seed length, width and number of seeds per capsule (Table II and Plates 1,2 and 3).

The Habit of *Bixa orellana* L. varied among the 2 accessions. Among the two accessions, Accession 1 is small tree and Accession 2 is medium sized tree (Plate 1).

The variations in leaf shape, size and colour differs in both the accessions. Accession 1 had cordate narrow leaves with dark green colour and accession 2 had cordate broad leaves with light green colour. Another marked difference was the flower, capsule colour and the shape. Accession 1 produced white coloured flowers with white stamens while Accession 2 produced rose coloured flowers with rose coloured stamens.

With regard to the capsules, variations were observed in shape, texture and colour. Accession 1 produced green coloured ellipsoid capsules with green spines and Accession 2 produced green coloured ovate capsules with green spines. Even the texture of the capsule coat exhibited variations.The accession 1 produced soft textured capsules while accession 2 produced rough textured capsules(Plate2).

The variation in seeds size and number of seeds present per capsules also differed in both the accessions. Accession 2 had more number of seeds -41 seeds per capsule than accession 1 -25 seeds per capsule (Plate 3).

B. Assessment of Intraspecific Genetic Diversity Using Single Sequence Repeat (SSR) Markers

The field of Plant breeding, plant genetics, biological diversity analysis, and cultivar identification routinely makes use of Molecular markers as they directly reveal genetic differences at the DNA level. Compared to other biomarkers, such as RAPDs and AFLPs, SSR markers are co-dominant, reproducible, multi-allelic and of low cost. SSR motifs are polymorphic, abundant, and randomly distributed in eukaryotic genomes a hence they have been widely used in studying genetic diversity between plant species (Hao, 2015).

The Amount of Genomic DNA present in both the accession of *Bixa orellana* L. was determined by using SpectraMax® i3x device. It was found that Accession 1(BU) had 261ng/uL of DNA and Accession 2 (Lalbagh) has 376ng/ul of DNA (Plate 4).

In the present study,10 SSR primer pairs were developed (Table I) and polymorphism was tested among two accessions of *Bixa orellana L*. Of them, 7 SSR primers showed polymorphism with

visible bands. A total of 43 discernible and reproducible SSR bands were generated by employing the aforementioned 7 SSR primers across the two accessions of *B. orellana* L. Out of them, 22 bands were polymorphic (49.6%). The number of bands produced per primer ranged 1-8 with an average of 4.3 bands per primer. Maximum number of bands was produced by the primer TBG-Centa F10, i.e. 4 bands and minimum number of bands were produced by primers TBG-Centa F1, TBG-Centa F3, TBG-Centa F8 i.e. 1 band. The primers TBG-Centa F15 and TBG-Centa F31 shows 100%polymorphism. The primers TBG-Centa F14, TBG-Centa F19 and TBG-Centa F26 did not show any amplification (Table III). The gel pictures of PCR amplicons with SSR primers with banding profile of both the accessions of *Bixa orellana* L. (Table IV- X and Plate 5-11).

C. Estimation of Total Pigment (Bixin) Content

The total Bixin content was estimated according to (Mc Kneown & Mark 1962). The significant differences in the pigment (Bixin) Colour and the amount were observed. The percentage of pigment yield was maximum of 1.2% in accession 2 Lalbagh and minimum of 0.63% in accession 1 Bangalore University (Table XI and Plate 12).

D. Statistical Analysis

The data analysed statistically for the total dye content for both the accessions of *Bixa orellana* L., was found to be highly significant at $p < .05$ (Table XII and Figure 1).

The tree with highest bixin content coupled with maximum seed production were termed as the elite variety. The studies on total dye content revealed that the accession 2 (Lalbagh) was considered to be elite due to its highest seed production and maximum yield of Bixin compared to accession 1 with lowest seed production and minimum yield of Bixin.

IV. DISCUSSION

Our studies showed that both the accessions of *Bixa orellana* L., collected from 2 different areas of Bangalore revealed wide variations in their morphology with respect to habit, branching pattern, flowering time, flower and stamen colour, fruiting period, capsule shape, size and its texture, seed length, width and number of seeds per capsule.

With respect to the studies on genetic diversity, ours is the first attempt to develop and utilize SSR markers to examine genetic diversity in *Bixa orellana* L., bearing white and rose coloured flower with green capsules and green spines. The SSR molecular markers used in this study revealed high levels of genetic diversity in both the accessions of *Bixa orellana* L.

With respect to seed bixin content, the accession-2 with Rose coloured flower with green capsule and green spines showed maximum of 1.20% than the accession 1 producing white flower with green capsule and green spines with 0.63% and hence accession 2 was the elite one.

Hence our studies are the first of its kind to report that it is always the combination of Rose or pink colour flowers with either red or maroon or green colour capsules bearing red or green spines is found to have maximum dye (bixin) content than white flowers and green capsules bearing green spines

The above said new 6th diverse accession of *Bixa orellana* L.,reported from lalbagh showed wide morphological variations (Poornima and Ambika,2014). But this 6th accession was not subjected to genetic analysis to find out the genetic relatedness of this accession to the other 5 divergent accessions of *Bixa orellana* L (Poornima,2006). Hence our present findings is the first of its kind to establish the morphological diversity, genetic diversity and dye diversity between the 6th accession collected from Lalbagh, Bangalore, Karnataka with the other 5 divergent accessions reported (Poornima, 2006 & Poornima et al, 2009).

CONCLUSION

We authors report for the first ever time in the literature that the cultivation and the multiplication of the above said elite combinations of *Bixa orellana* L., accessions would lead to the improved productivity and thus the net economic returns of the same will ensure a profitable enterprise.

Hence, the maximum availability of this natural food grade colourant will reduce the demand for its synthetic counterparts contributing for the improvement of human health and the environment at large.

ACKNOWLEDGEMENTS

We authors extend our sincere thanks to Dr. Shivashankar, Chairman, Department of Life Science, Bangalore University for the facilities extended and the Skanda life Sciences Pvt. Ltd., Bangalore for their timely help in designing and helping us to Conduct genetic diversity studies Using SSR Markers.

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Sl. No.	Seq. ID	Seq. Name	Seq. Text	Con. pm/µl	Tm °C	GC %	Mol. wt. Da	ODU
1	24900	TBG-Centa F1	For-AGGACTTGACACTGCTTTTGCT	699.4	53.0	45.5	6716.4	31.7
	24901	TBG-Centa F1	Rev-TGCCTTCTCCTTCTTCATCTTC	749.7	53.0	45.5	6529.2	28.5
2	24902	TBG-Centa F2	For-CTACTCTATCCCGCAAATCCTT	726.6	53.0	45.5	6565.3	31.5
	24903	TBG-Centa F2	Rev-CTCTCTCTCGTTTCTCGCC	861.1	53.2	57.9	5632.6	27.5
3	24904	TBG-Centa F3	For-AGTGTTGATGATGATGACGAGG	599,4	53.0	45.5	6894.5	30.7
	24905	TBG-Centa F3	Rev-CAGACTCATTTGCTTTTGCTTG	714.1	51.1	40.9	6682.3	31.0
4	24906	TBG-Centa F8	For-AGAATCAATACATACAGCCCCG	607.4	53.0	45.5	6681.4	30.6
	24907	TBG-Centa F8	Rev-AAACGAAAGATTGTGAGAAGGG	545.1	51.1	40.9	6905.6	30.6
5	24908	TBG-Centa F10	For-CCAAAACCATTCTCTCCACTTC	675.3	53.0	45.5	6534.2	29.6
	24909	TBG-Centa F10	Rev-CTCTTCTTTGTCGCCATCTTCT	811.5	53.0	45.5	6569.2	31.5
6	24910	TBG-Centa F14	For-TCCTCCAAAATACCACCATACC	502.1	53.0	45.5	6552.3	23.4
	24911	TBG-Centa F14	Rev-GACCAATGAGTGCCAAAAGAAT	579.9	51.1	40.9	6785.5	31.1
7	24912	TBG-Centa F15	For-GAACTTTCGCCTCTTCTCTTGA	711,1	53.0	45.5	6627.3	30.1
	24913	TBG-Centa F15	Rev-TCCTCATTTATCTCCCTCGGTA	730.3	53.0	45.5	6587.2	30.3
8	24914	TBG-Centa F19	For-TTAGCATTTAGAAGGTCAGGGC	622.9	53.0	45.5	6814.5	30.9
	24915	TBG-Centa F19	Rev-ATTTACAGCAATCAGAGACGCA	512.8	51.1	40.9	6736.4	26.4
9	24916	TBG-Centa F26	For-ATGGGAGAGAAATAAAGGAGCC	504.3	53.0	45.5	6890.6	28.2
	24917	TBG-Centa F26	Rev-GAAACGATAGTCAGGGATTGGA	595.1	53.0	45.5	6872.5	31.6
10	24918	TBG-Centa F31	For-AGAGCACACCTTTATCCCTTTG	680.3	53.0	45.5	6645.3	30.6
	24919	TBG-Centa F31	Rev-AGAAGAAGAAGGAGGATTTGGG	515.2	53.0	45.5	6961.6	28.9

Table I. Description of 10 *Bixa orellana* L. SSR markers including their Sequence ID, Sequence names, length(bases), concentration (pm/µl), temperatures (Tm), GC content (%), molecular weight (Da) and ODU (Optical Density Units).

AREA OF COLLECTION			
Sl.NO.	PARAMETERS	ACCESSION 1 (BANGALORE UNIVERSITY)	ACCESSION 2 (LALBAGH)
1	HABIT(SIZE)	SMALL TREE	TREE
2	HEIGHT OF THE TREE	3.4 METER	7.3 METER
3	BRANCHING PATTERN	FROM BASE	FROM BASE
4	NUMBER OF BRANCHES	15	25
5	LEAF SHAPE	CORDATE(NARROW)	CORDATE(BROAD)
6	COLOUR OF THE LEAF	DARK GREEN	LIGHT GREEN
7	FLOWERING TIME	JULY	FEBRUARY & APRIL
8	FLOWER AND STAMEN COLOUR	WHITE	ROSE AND ROSE
9	FRUITING PERIOD	AUGUST	MARCH & MAY
10	CAPSULE COLOUR	GREEN	GREEN
11	CAPSULE SHAPE	ELLIPSOID	OVATE
12	CAPSULE LENGTH	5.0 CM	4.5 CM
13	CAPSULE BREADTH	4.5 CM	4.0 CM
14	CAPSULE TEXTURE	SOFT	ROUGH
15	SEED LENGTH	0.4 MM	0.5 MM
16	SEED WIDTH	0.3 MM	0.4 MM
17	NUMBER OF SEED PER CAPSULE	25	41

Table II. Morphological studies of two accessions of *Bixa orellana* L. Collected from 2 different areas of Bangalore, Karnataka (Accession 1: Bangalore university, Accession 2: Lalbagh).

1(A) 2(A)

ACCESSION 1 **ACCESSION 2**

Plate 1: Habit of *Bixa orellana* L. Accessions collected from2 different areas of Bangalore, Karnataka (Accession 1: 1(a) Bangalore university, Accession 2: 2(a) Lalbagh).

1(b)

1(c)

1(d)

1(e)

ACCESSION 1

2(b)

2(c)

2(d) **2(e)**

ACCESSION 2

Plate 2: Variation in habit, leaf shape, size& colour, Flower colour, Capsule colour (Fresh and Dry) of *Bixa orellana* L., accessions collected from 2 different areas of Bangalore, Karnataka (Accession: 1: 1(b), 1(c),1(d), 1(e) Bangalore university, Accession 2: 2(b), 2(c),(d), 2(e) Lalbagh).

1(f) **2(f)**

ACCESSION 1 **ACCESSION 2**

Plate 3: Variation in capsule size and shape of *Bixa orellana* L., accessions collected from 2 different areas of Bangalore, Karnataka (Accession 1:1(f) Bangalore university, Accession 2: 2(f) Lalbagh).

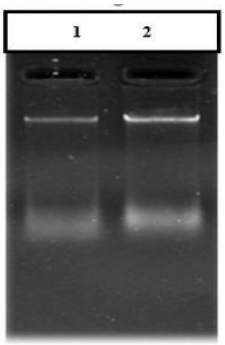

Plate 4: Genomic DNA isolation from leaves of both the accessions of *Bixa orellana* L. Lane 1- Accession 1 (Bangalore University), Lane 2- Accession 2 (Lalbagh).

Table III. Percentage polymorphism of both the accessions of *Bixa orellana* L. Analysed using different SSR primers.

Primers	Total number of bands	No. of polymorphic bands	% of Polymorphic bands
TBG-Centa F1	4	1	25
TBG-Centa F2	8	2	25
TBG-Centa F3	3	1	33.3
TBG-Centa F8	7	1	14.2
TBG-Centa F10	8	4	50
TBG-Centa F15	8	8	100
TBG-Centa F31	5	5	100
TBG-Centa F14	no amplification	no amplification	-
TBG-Centa F19	no amplification	no amplification	-
TBG-Centa F26	no amplification	no amplification	-
Total	43	22	

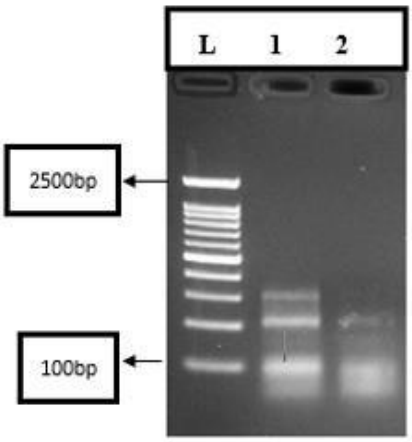

Plate 5: PCR amplicons with SSR primer of two accessions of *Bixa orellana L.* Of TBG-Centa F1
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

1	**0**
1	**1**
1	**1**
1	**1**

Table-IV: Banding profile of primer TBG-Centa F1 for the two accessions of *Bixa orellana* L.

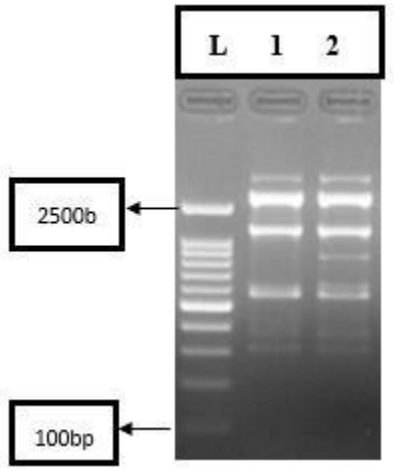

Plate 6: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of TBG-Centa F2
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

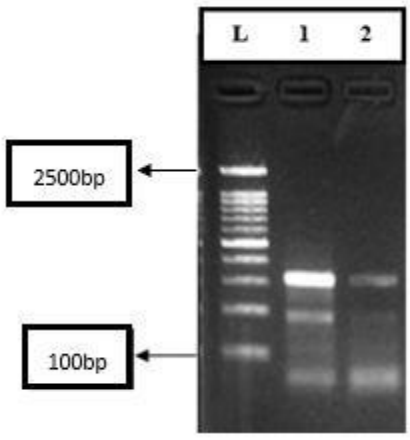

Plate 7: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of TBG-Centa F3
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

1	**1**
1	**1**
1	**1**
0	**1**
0	**1**
1	**1**
1	**1**
1	**1**

Table-V: Banding profile of primer TBG-Centa F2 for the two accessions of *Bixa orellana* L.

1	**1**
1	**0**
1	**1**

Table-VI: Banding profile of primer TBG-Centa F3 for the two accessions of *Bixa orellana* L.

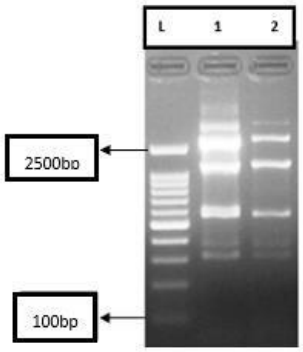

Plate 8: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of TBG-Centa F8
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh)

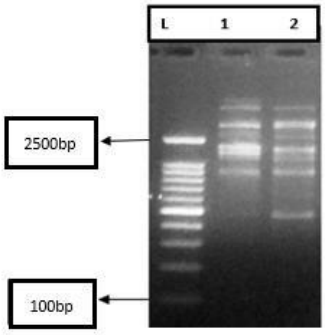

Plate 9: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of TBG-Centa F10
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

1	**0**
1	**1**
1	**1**
1	**1**
1	**1**
1	**1**
1	**1**

Table-VII: Banding profile of primer TBG-Centa F8 for the two accessions of *Bixa orellana* L.

1	**1**
1	**1**
0	**1**
1	**1**
0	**1**
1	**1**
0	**1**
0	**1**

Table-VIII: Banding profile of primer TBG-Centa F10 for the two accessions of *Bixa orellana* L

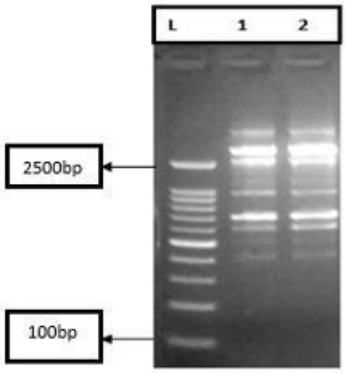

Plate 10: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of tbgcenta F15
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

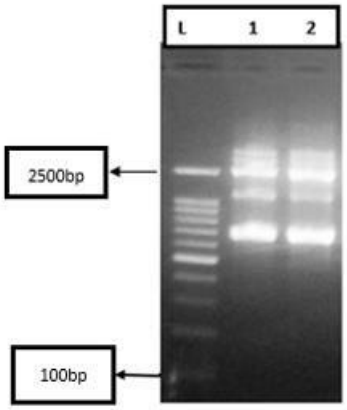

Plate 11: PCR amplicons with SSR primer of two accessions of *Bixa orellana* L. Of tbgcenta F31
L- Ladder, Lane 1- Accession 1 (Bangalore University).
Lane 2- Accession 2 (Lalbagh).

1	**1**
1	**1**
1	**1**
1	**1**
1	**1**
1	**1**
1	**1**
1	**1**

Table-IX: Banding profile of primer TBG-Centa F15 for the two accessions of *Bixa orellana* L.

1	**1**
1	**1**
1	**1**
1	**1**
1	**1**

Table-X: Banding profile of primer TBG-Centa F31 for the two accessions of *Bixa orellana* L

Sl.No.	PARAMETERS	ACCESS1ON 1	ACCESSION 2
1	COLOUR OF THE BIXIN EXTRACT	YELLOW	ORANGE
2	TOTAL PIGMENT BIXIN (%)	0.63	1.29

Table XI. The quality and the total pigment content of the dye from seeds of *Bixa orellana* L., accessions collected from 2 different areas of Bangalore, Karnataka (Accession 1: Bangalore university, Accession 2: Lalbagh).

Plate 12: Variation in the Bixin colour from seeds of *Bixa orellana* L., accessions Collected from 2 different areas of Bangalore, Karnataka (Accession 1: Bangalore university, Accession 2: Lalbagh).

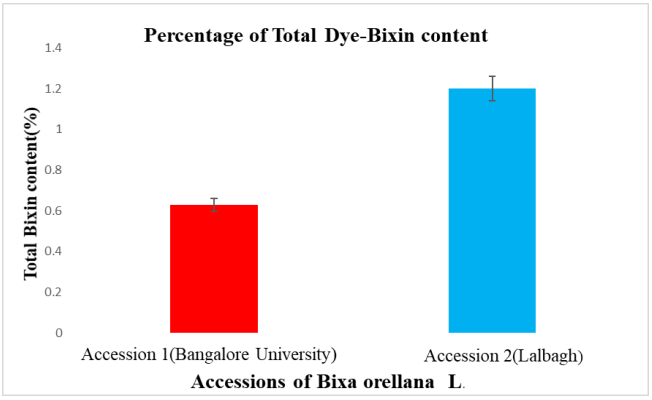

Fig.1. Total pigment (Bixin) content (%) of *Bixa orellana* L., accessions collected from different areas of Bangalore, Karnataka (Accession 1: Bangalore university, Accession 2: Lalbagh).

PARAMETERS	VALUE
Mean	0.92
SD±	0.32
T-Value	-8.6368
P-Value	0.000494
Significance	Yes

Table XII. Statistical data for seed dye content for both the accessions of *Bixa orellana* L.

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