IMAGE: A MAP OF THE STARS OF THE ORION CONSTELLATION

Print ISSN: 2631-8490 Online ISSN: 2631-8504

JournalPreview

London Journal of Research in Science: Natural and Formal
Volume 22 | Issue 7 | Compilation 1.0

London Journals Press

Journal Content
In this Issue

London Journals Press

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Classification: DDC Code: 813.54 LCC Code: PS3553.H15

Language: English

London Journals Press

LJP Copyright ID: 925671
Print ISSN: 2631-8490
Online ISSN: 2631-8504

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The research study reports a fresh synthesis of clay-biochar nanocomposite material derived from natural clay and Prosopis Juliflora plant for effective removal of lead metal from aqueous solution. The nanocomposite material was formed by heat pyrolysis of clay and biochar biomass at different temperatures and synthesis was confirmed by characterization with XRF, EDX, FTIR, XRD, and SEM. The characterization revealed successful impregnation of the clay minerals on the surfaces of the biochar material to form the composite material. The efficacy of removal of lead metal by the composite material was ascertained by the batch adsorption method. The three materials of calcined clay, biochar, and composite material all produced a good removal efficacy. Adsorption isotherms of Frendluich and Langmuir to study adsorption were used which showed near to fit adsorption isotherms for lead removal from aqueous solution. The data also showed a pseudo-second-order reaction for the removal of lead metal.

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I. INTRODUCTION

All forms of life on earth depend on water to exist in the ecosystem and it is one of the most essential things for human survival (Obinnaa & Ebere, 2019). Fast population growth, industrialization, and unplanned urbanization are major causes of severe water pollution and surrounding soil. Discharges of toxic industrial and untreated sanitary waste, and runoffs from agricultural land can be major causes of freshwater pollution. Toxic pollutants released into wastewater can be harmful to aquatic organisms, which also make normal water bodies unsuitable for consumption. Studies show that it is a major cause of death and illness around the world killing 1.8 million people in 2015. This makes water pollution a global issue and requires continuous assessment and revision of water resources policies at all levels. The effects of pollution are greater in shallow, confined, or slow-moving streams. Excessive use of fertilizers, herbicides, and pesticides can be life-threatening if washed into the river by rain. Excessive phosphorus in fertilizer leads to serious eutrophication. (Owa, 2014).

Heavy metals are natural elements with a high atomic weight and a density more than five times that of water (Duffus, 2002). Heavy metals are considered trace elements because they are present in various environmental matrices at trace concentrations (ppb range less than 10 ppm). Their bioavailability is affected by physical factors such as temperature, phase association, adsorption, and isolation. Heavy metal pollution in the aquatic environment is a concern for the global environment due to their toxic effects and accumulation through the food chain. Various

regulatory agencies have set maximum regulatory limits on the emission of toxic heavy metals into water systems. Allowed maximum limits in drinking water for lead metal by different local and international organizations are shown in Table 1 (U.S.EPA, 2009; WASREB, 2006; WHO, 2011; EU, 2018; BIS, 2012).

Table 1: Allowed maximum limits in drinking water for lead ion by different local and international organizations

Organization	Pb (mg/L)
USEPA, EU, WHO, BIS	0.01
KEBS/NEMA/WASREB	0.05

Nevertheless, metal ions are added to the water stream at concentrations much higher than the limits regulated by industrial activities, causing health hazards and environmental pollution. (WHO, 2011). A study of open sewerage in Nairobi, Kenya, showed that sewage and soil samples from open sewerage in the industrial area of Nairobi contained more than the minimum acceptable levels of heavy metals (Kinuthia et al., 2020). Based on WHO, EU and KEBS standards for drinking water, Nairobi Dam water is heavily contaminated with Pb, Cd, Cu and Ni and therefore, it is water is not suitable for human or animal consumption (Ndeda & Manohar, 2014). Some of the health effects of lead include damage to the brain, kidney, sperm damage, increase in blood pressure, abortion and miscarriages, reduced learning abilities in children, nervous system disruptions, disruption in the synthesis of hemoglobin and anemia to name a few (Halim et. al., 2003). Lead bioaccumulates in the bodies of water and soil organisms. The acceptable maximum limit for lead in drinking water is 0.01 mg/L according to USEPA, EU, WHO, and BIS standards.

There are usually several methods used to remove various heavy metal ions. Some of this include: ion exchange, reverse osmosis, ultrafiltration, electrodialysis, precipitation and adsorption process among others. Adsorption is an effective separation process due to its low operating cost and low energy consumption. Moreover, use of natural and green chemistry sources of adsorbents which is assumed low-cost and abundant in nature has gained prominence in the recent research studies of heavy metal remediation. Clay has recently gained prominence as a remediation and clean-up tool for various environmental contaminants including heavy metals (Bhattacharyya et. al, 2006). The usage of natural clay in the remediation of heavy metals has been employed because of its cost- effectiveness (Sdiri et. al, 2012). Due to widespread accessibility and cheaper cost of naturally occurring clay it has become an attractive adsorbent in the remediation of heavy metals from contaminated sources. Biochar is a fine-grain, porous and carbonaceous solid that is produced from waste biomass remains under circumstances with limited oxygen content and low to medium temperatures (450-650°C) by slow pyrolysis (Lehmann & Joseph, 2012). Biochar is a highly porous structure with a large surface area, a high pH, abundant surface functional groups, high cation exchange capacity (CEC), has adsorption ability, contains organic matter, and has high water holding capacity. A composite is a natural or manufactured material composed of two or more materials with dissimilar physical and biochemical properties that are separate and distinct at macroscopic and microscopic scales within the substance (Srinivasan, 2011). Various nanocomposites have been used as adsorbents in heavy metals removal from various matrices in the environment. Kanchana et al., (2012) concluded that the nanocomposite material of chitosan composed of methylcellulose and nanochitosan with kaolin clay in the existence of cross-linking agent performs as a noble adsorbent to eliminate Pb (II) ions from artificial wastewater.

The study presents a novel synthesis procedure for clay-biochar composites material for effective removal of lead ions from aqueous solution. The Biochar was derived from *Prosopis Juliflora* from Northern Kenya.

II. MATERIALS AND METHODS

2.1 Study Area

Natural clay was collected from the Kimathe Valley in the Mukurweini sub-county, Nyeri county (latitude 0° 37' 55.9 "S, longitude 37° 9' 43.8' 'E) as shown in Figure 1. Random samples were taken from three locations. The collected samples of clay were taken at 500 m intervals in the same area where the clay mines were located. The depth of the samples taken from the surface was 0.45m. The samples were packed in a plastic container then taken to the laboratory for analysis. The sampling of *Prosopis Juliflora* was done purposively on the banks of river Tana, in Garissa county (latitude 0° 27' 50" S, longitude 39° 38' 12' 'E) as shown in Figure 2. The types of *Prosopis* tree were selected from possibly clean zones as wood biomass supplies for charcoal production. *Prosopis Juliflora* trees were carefully selected by their similar age (which was arrived at by diameter of tree stem), evading defects in trees and possibility of pollution (sampling was done at a distance of approximately 100–150m from any road network and 2000 m from any industrial pollution source).

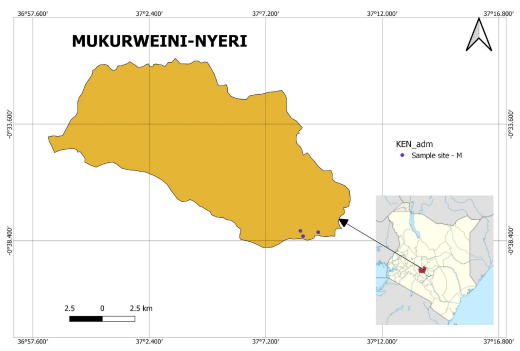


Figure 1: Sampling area in Mukurweini in Nyeri County

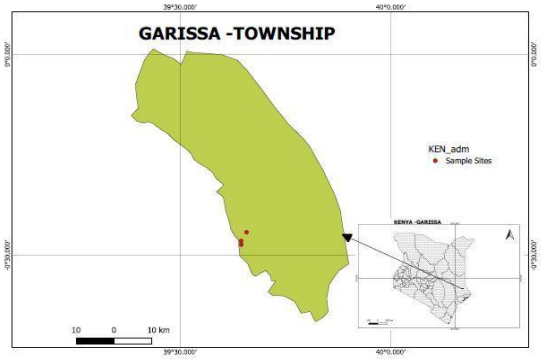


Figure 2: Sampling area in Garissa town in Garissa County

III. MATERIALS AND METHODS

The calcination of the clay was done with a furnace (Daihan FHX, Digital Muffle Furnace, Standard-type, 1200°C, FHX-03/05/12/ 14/27/63) at calcined at 1000 ˚C for a period of one hour. The cut pieces of *Prosopis* were grounded and a fine particle of the ground powder was air-dried for 24 hours to reduce the moisture content. The pyrolysis process was carried out with a furnace (Daihan FHX, Digital Muffle Furnace, Standard- type, 1200 °C, FHX-03/05/ 12/14/27/63). 5g of the feedstock was placed in a ceramic crucible and subjected to heat pyrolysis at different temperatures (200 °C, 400 °C, 500 °C, and 700 °C respectively) for 2 hrs. After the process of pyrolysis was over the furnace was left for some time for the sample to cool to room temperature. The biochars obtained were marked as S-1-1, S-1-2, S-1-3, and S-1-4 for sample S-1, and S-2-1, S-2-2, S-2-3, and S-2-4 for sample S-2 representing 200 °C, 400 °C, 500 °C, and 700 °C respectively as shown in Table 2.

Table 2: Bio-char samples ID at various temperatures

Temperature sample ID	200°C	400°C	500°C	700°C
S-1	S-1-1	S-1-2	S-1-3	S-1-4
S-2	S-2-1	S-2-2	S-2-3	S-2-4

The modified calcined clay obtained and bio-char were fused by heat pyrolysis using a ratio of 1:1 to form a nanocomposite material. Initially, equal amounts of each were used and subsequently varying ratios to achieve an optimum dispersion for impregnation. For the adsorption studies, an equal amount of the clay silicates and biochar (5g each) was used.

IV. CHARACTERIZATION

The calcined clay, biochar and nanocomposite materials were characterized using FTIR (Nicolet 6700 FTIR system, Model: 16F PC), TEM(JEM-2100F), SEM (Model; Quarto S), XRF, EDX, and XRD (Rigaku powder XRD-model ultima IV with conditions of; start angle 5 and stop angle 70; scan speed 5). Elemental analysis of the calcined clay, biochar and nanocomposite materials were done with XRF and EDX. X-ray diffraction of biochar and composite material were performed to determine the phase analysis. Morphology investigation and measurement of size was carried out by Field Emission Scanning Electron Microscopy (FESEM), and Transmission Electron Microscopy (TEM). For FTIR analysis pellets were made using KBr for the analysis.

V. METHOD OF BATCH ADSORPTION

Batch adsorption procedure was used to study the removal efficacy of lead by the calcined clay, bio-char, and nanocomposite materials. 1.000g of lead metal strip/wire (99.99%) purity was dissolved in nitric acid and diluted to 1 liter to give 1000 mg/L. Standard of lead metal (1000 ppm) was diluted in a 1L volumetric flask to achieve a concentration of 1ppm of lead. 25 mL volume of the diluted solution was put in the Erlenmeyer flask and appropriate dosage of the synthesized material were added. pH adjustment was carried out using dilute NaOH or HCl to achieve desired pH. The extent of removal of lead was evaluated by varying different parameters like pH, interaction time, and adsorbent amount (Ahmed et al., 2006). Before the analysis, the samples of adsorption study of different synthesized material for the removal of lead was subjected to filtration using nylon syringe filters with a pore size of 0.22 µm and a diameter of 13 mm from the membrane solution. Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used to analyze the concentration of the lead metal ion before and after the adsorption procedure. Batch adsorption procedure was used to study the efficacy of the composite material. Basic quality control and assurance, sample triplicates, sample blanks, and calibration standards protocols were followed. The removal efficiency was calculated based on the reducing concentrations of lead in water of each sample using Equation 1.

$$\text{Percent removal efficiency} = \frac{Ci - Cf}{Ci} \times 100 \quad (1)$$

Where C_i is the initial concentration of the heavy metal (mg/L) and C_f is the final concentration of the heavy metal in water after the adsorption process (mg/L).The data obtained from experiments were used to test the applicability of various isotherms like Freundlich and Langmuir isotherm models. Pseudo-first-order kinetics and second-order kinetics equations were used to study the involved adsorption kinetics (Sazali et al., 2020).

VI. RESULTS AND DISCUSSION

6.1 Elemental analysis

Elemental analysis of the composition of calcined clay was determined by XRF and EDX. The results showed that the clay was composed mainly of silicates, aluminate and iron among others as shown in Table 3. The elemental analysis of the biochar was done by EDX. Table 4 shows the EDX elemental analysis of the biochar which indicated that it is composed of carbon and oxygen which was consistent with what was reported in the

literature of most biochars. This information corresponded well with the EDX analysis as seen in Figure 3. The presence of gold (Au) is attributed to the FESEM instrument used which was coupled with EDX and usually gold is used for sputter coating material in the initial stage of sample preparation.

Table 3: Percentage composition of calcined clay using XRF

Composition	%
Al_2O_3	32.4
SiO_2	55.0
K_2O	1.1
CaO	1.1
Fe	6.1
P_2O_5	0.5

Table 4: Percentage elemental composition of *Prosopis Juliflora* biochar at 500°C

Element	Wt. %
Carbon (C)	62.5
Oxygen (O)	21.1
Potassium (K)	6.0
Calcium (Ca)	5.3
Chloride (Cl)	2.3
Sodium (Na)	1.0

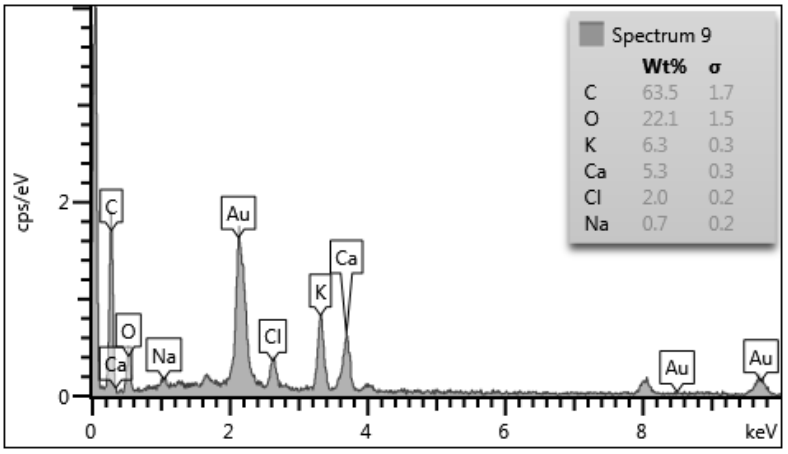


Figure 3: EDX patterns of *Prosopis Juliflora* biochar at 500 °C

The elemental analysis of the nanocomposite material was done with EDX. The results as shown in Figures 4 (A) and (B) showed the elemental composition of the nanocomposite to be high in carbon (C), oxygen (O), silica (Si), aluminium (Al), and iron (Fe). The carbon came from the biochar while the other elements originated from clay material. The average composition was 49.05 % C, 35.85 % O, 5.7 % Si, 5.2 % Al and 2.05 % Fe.

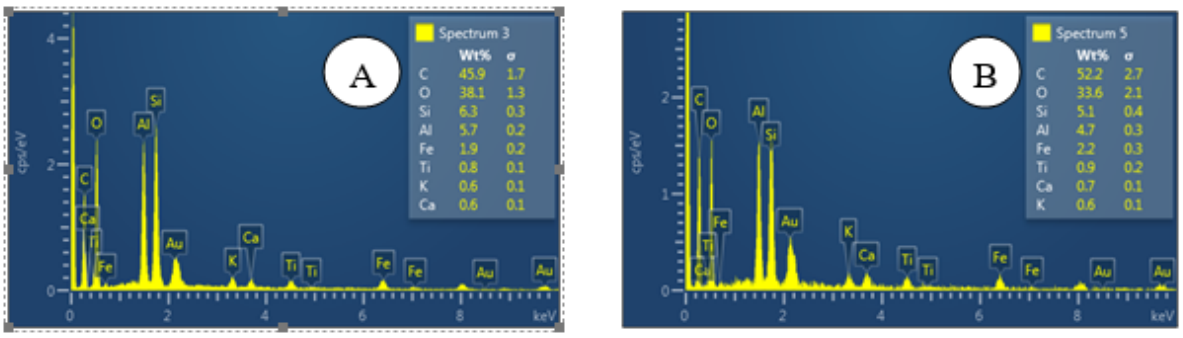


Figure 4: EDX patterns of the nanocomposite material of duplicate (A) and (B)

FTIR

The FTIR spectrum of *Prosopis* Biochar in Figure 5 shows the O-H stretching vibrations of hydrogen-bonded hydroxyl groups at 3500 cm^{-1} bands and the CO_2 absorption peak at 2,350 cm^{-1}. The peak at 1,600 cm^{-1} was attributed to carboxylate (COO^-) and primary amine N-H bending, (Liu et al., 2015), and ~1,405 cm^{-1} was ascribed to aromatic C=C stretch (Zhao et al., 2017). The band around 1,099 cm^{-1} was ascribed to C-O stretching vibrations or the C-N stretch of an aliphatic primary amine (Coates, 2006).

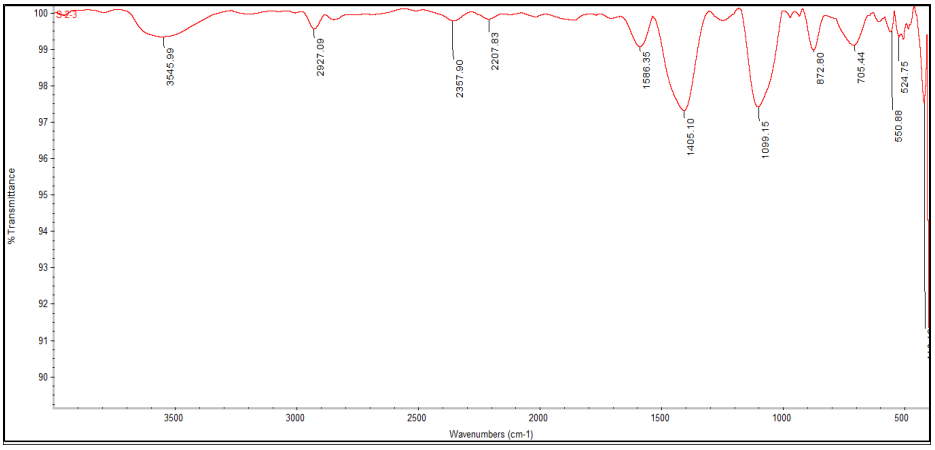


Figure 5: FTIR spectrum of *Prosopis Juliflora* biochar at 500 ° C.

The FTIR spectrum of the nanocomposite material is shown in Figure 6. The spectrum showed a broad weak peak at approximately 3645 cm^{-1} symbolic of O-H stretching as a result of the hydroxyl group and another peak at 2349 cm^{-1} associated with CO_2 absorption. The band at 1,063 cm^{-1} was ascribed to C-O stretching vibrations. This was an indication that the biochar material composition was well contained in the nanocomposite material, therefore successful impregnation of clay silicates on the biochar surface.

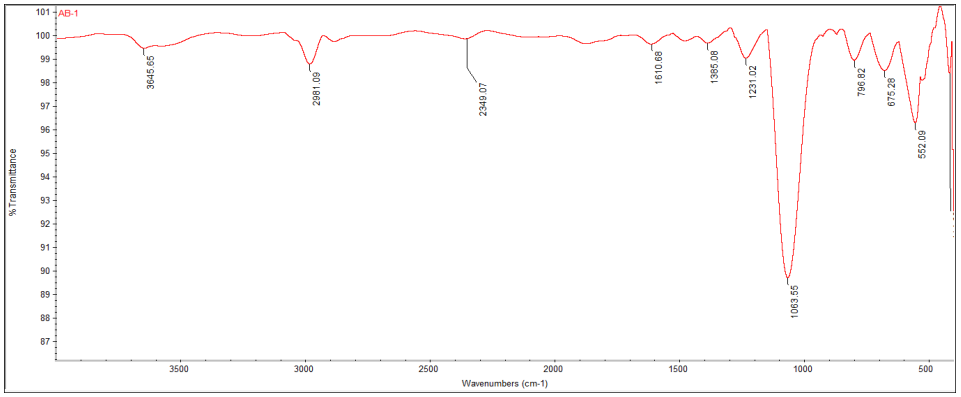


Figure 6: FTIR Spectrum of Nanocomposite Material

XRD

The X-ray Diffraction (XRD) pattern of *Prosopis* biochar is shown in Figure 7. The existence of cellulose or other related organic compounds was shown by the rise in the background level and by a huge hump between 11 and 13° (Fancello et al., 2019). The broad peak at the 2θ values around 23° was related to the crystalline cellulose in the spectrum (Osman et al., 2018). A narrow sharp peak at around 30° was seen and recognized as amorphous carbon (Fu et al., 2016). XRD analysis of clay-biochar nanocomposites showed the existence of mineral crystals. In the spectrum, the three strong peaks at 19.9°, 25°, and 35° were identified as expansible phyllosilicates (Yao et al., 2011) as shown in figure 8. These XRD results agreed well with EDX results that the pyrolysis method had successfully implanted silicates onto the carbon surfaces of the biochar matrix to form a clay-biochar nanocomposite.

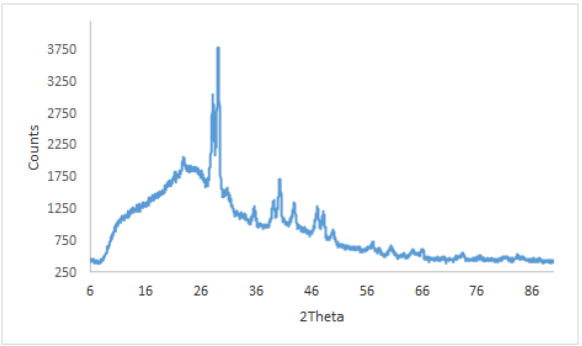


Figure 7: FTIR spectrum of *Prosopis Juliflora* biochar at 500 °C.

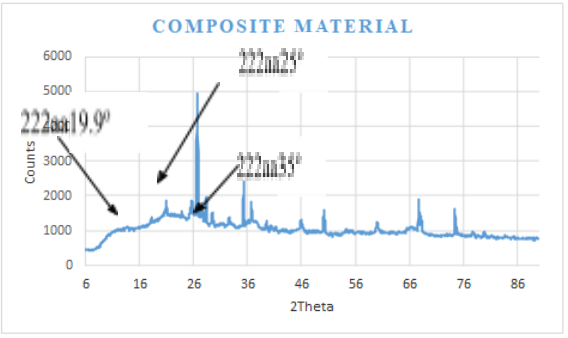


Figure 8: XRD pattern of clay-biochar nanocomposite

SEM and EDX

The images from SEM of biochar revealed two key morphological structures for samples: fibrous structures and pith. As can be seen from Figures 9A, 9B, and 9C the biochar sample was described by rough particles of different sizes with vascular features packed in rolls and had a comparatively flat surface, this had similarly been observed in literature (Zhang et al., 2014; Wang et al., 2015).

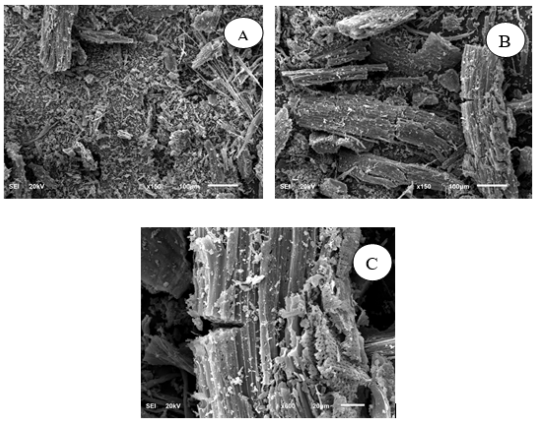
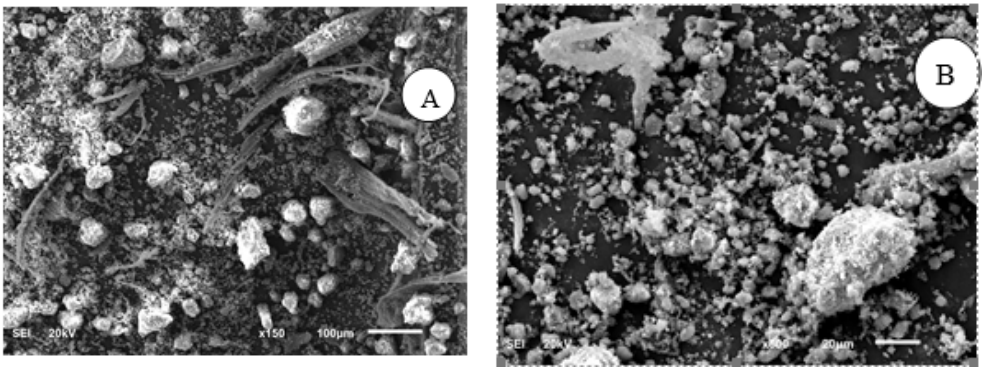


Figure 9: SEM images of the *Prosopis Juliflora* biochar at (A): 100 µm, (B): 100 µm, and (C): 20 µm

The SEM images of the clay biochar composites revealed that the sample surface was mainly covered by thin-film structures as shown in Figure 10 (A). At a higher magnification (X5), the films showed layered surfaces as seen in Figure 10 (B). Structural morphology of calcined clay gave the description as shown in Figure 10 (C) (Wang et al., 2004). The surface covered with clay particles on the biochar was additionally established by the EDX analysis as given in Figure 11. Both the EDX spectrum and the SEM image of the surface revealed high peaks for silicon, aluminum, titanium and iron, all of which are typical of the elemental composition of clay. The SEM images of clay revealed clear morphology of typical clay and the composite displayed successful impregnation of biochar on the surface of the clay minerals as confirmed by EDX analysis.



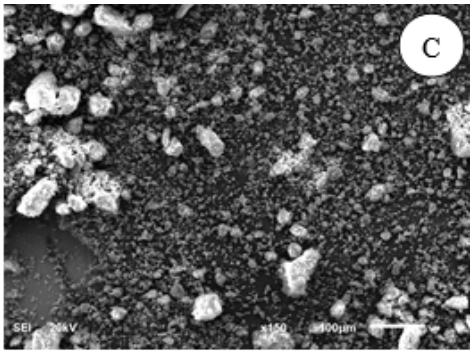


Figure 10 (A-C).: (A): SEM images of nanocomposite at 100 µm; at 20 µm (B) and of calcined clay (C)

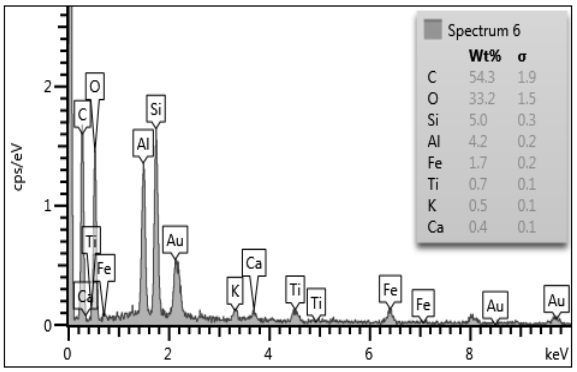
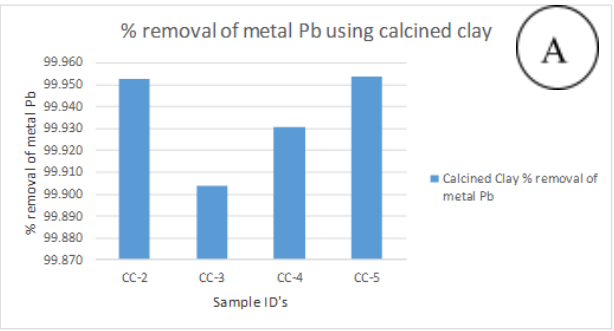


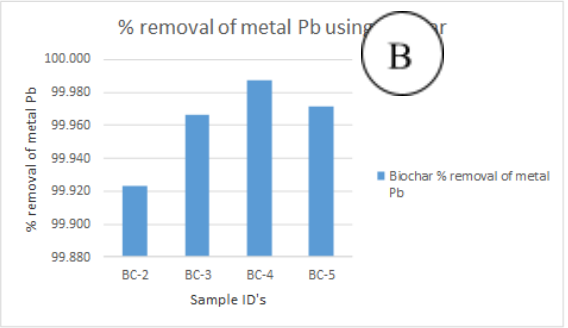
Figure 11: EDX spectrum of the nanocomposite

4.2 Adsorption and efficacy of the composite material

During the batch adsorption analysis, the following parameters were taken into account: pH, contact time, speed of the shaker, and dosage of the material. The data from the adsorption results was not varying for the four heavy metals and the maximum removal was realized after 60 mins of contact time for all the metals which agreed with Rediske (2014). The pH range was between pH 4 – pH 9. The highest removal as seen from the adsorption results at pH 8 was 99.95% removal efficiency for Pb. Therefore, pH 8 was selected as the optimum pH for adsorption of lead ion for the rest of adsorption experiments. For the speed of the shaker, the results from the adsorption for the heavy metals were performed ranging from 50 rpm to 200 rpm using an orbital shaker and maximum efficiency was at 150 rpm.

The removal efficacy of calcined clay for lead ion from aqueous solution was at 99.35 % while biochar was at 99.6 % and nanocomposite material was at 99.8 % (12 (A-C)). Figure 12 (D) showed a summary of the percentage removal of lead ion by calcined clay, biochar and composite material from aqueous solution.





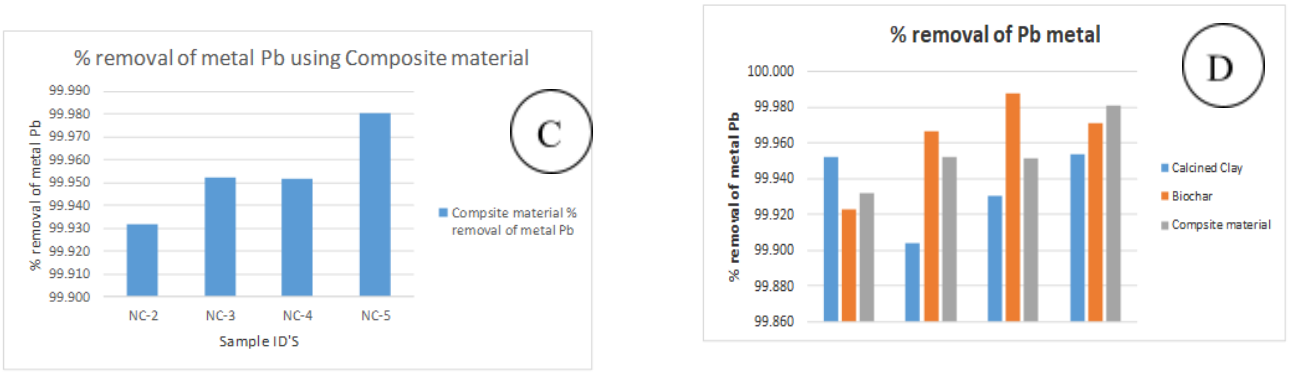
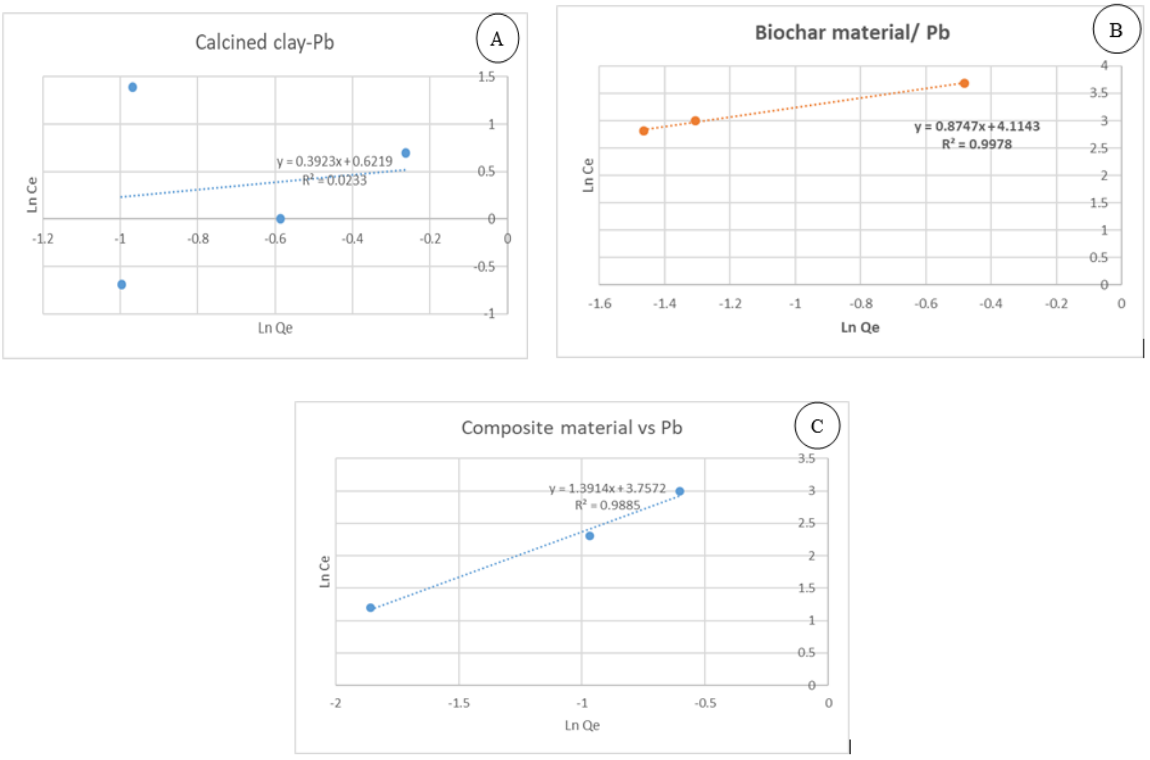


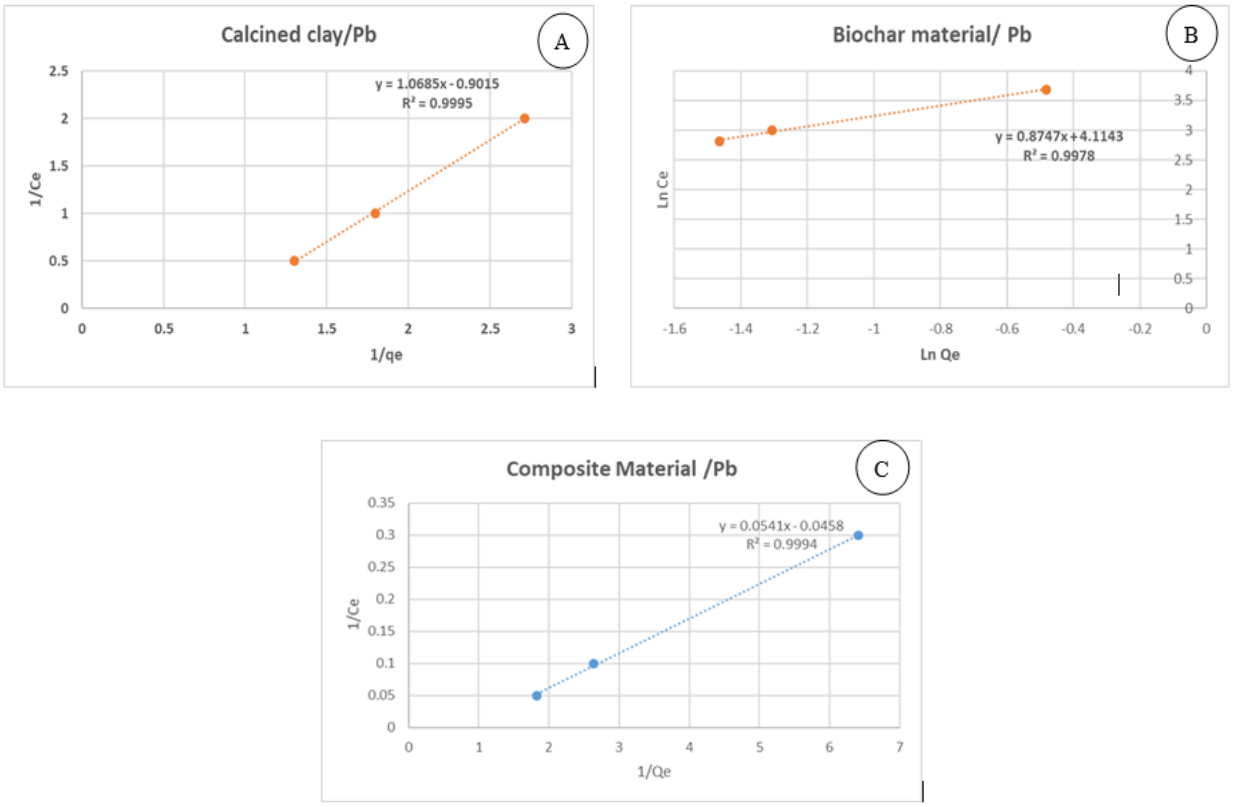
Figure 12 (A-D): removal efficacy of A: calcined clay; B: Biochar; C: composite material and D: summary of % removal of calcined clay, biochar and composite material

4.5 Freundlich and Langmuir isotherms

For Freundlich isotherm adsorption data, calcined clay removal for lead ion gave a poor fit with R^2 of 0.0233, while removal with biochar material had a fit of 0.9978 and composite material had a fit of R^2 of 0.9885 as can be seen in Figures 13(A-C). For Langmuir isotherm adsorption data calcined clay and composite material produced a perfect fit with R^2 of 0.999 while biochar had a fit of R^2 of 0.973 as can be seen in the Figures 14 (A-C).



Figures 13(A-C): Freundlich isotherms for A: calcined clay; B: Biochar and C: Composite material for removal of lead metal from aqueous solution.



Figures 14(A-C): Langmuir isotherms for A: calcined clay; B: Biochar and C: Composite material for removal of lead metal from aqueous solution

Tables 4 and 5 summarizes the calculated constants for both Freundlich (K_f, n and r^2) and Langmuir (q_m, K_L and r^2) isotherms

Table 5: Freundlich isotherms constants (K_f, n and r^2) for calcined clay, biochar and composite materials for lead metal

Materials	Freundlich Constants		
	K_f	n	r^2
Calcined Clay	0.131	0.824	0.0233
Biochar	14.763	2.359	0.9978
Composite	2.837	1.349	0.9885

Table 6: Langmuir isotherms constants (K_f, n and r^2) for calcined clay, biochar and composite materials for lead metal

Materials	Langmuir Constants		
	K_L	q_m	r^2
Calcined Clay	-36.30	0.151	0.9995
Biochar	19.25	9.116	0.9978
Composite	9.32	2.999	0.9994

The kinetic experiments indicated that the second-order kinetic pseudo-model fits the data better than the first-order pseudo-model. The second-order pseudo model gave straight-line fits while the first-order pseudo model did not. The slope and y-intercept from the linear regression were used to calculate the rate constant for the second-order experiment. The pseudo- second-order kinetics model is explained from a time vs. t/qt linear plot and the resulting parameters are as shown in Figure 13.

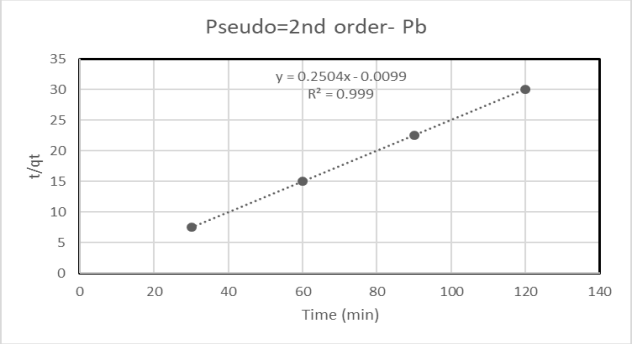


Figure 13: Pseudo second order plot of lead metal

V. CONCLUSION

The study focused on the synthesis of clay-biochar nanocomposite materials derived from clays and *Prosopis Juliflora* feedstock to effectively remove lead ion from aqueous solutions**.** Nanocomposite material was prepared by pyrolysis of clay and biochar biomass at different temperatures, and the synthesized material was characterized by XRF, EDX, FTIR, XRD, and SEM techniques to determine appropriate synthesis. Characterization revealed that the surface of biochar biomaterials was successfully impregnated with clay minerals in the formation of composite materials. The batch adsorption method was used in the study for the removal efficiency of the composite material of the lead ion. All three materials of calcined clay, biochar and nanocomposite produced impressive removal efficiency of lead ions from aqueous solution. Adsorption isotherms of Frendluich and Langmuir were used to study adsorption which confirmed close fit adsorption isotherms for lead elimination from aqueous solution. The results additionally confirmed a pseudo-2nd order reaction for the elimination of lead ion.

Funding support

We wish to appreciate the National Research Fund (NRF-Kenya) for the financial support of the Project.

Conflict of interest

All authors declare no conflict of interest

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Laundry Detergents: A Potential Resource of Pollution and Overutilisation

Aligina Anvitha Sudheshna & Dr. Meenu Srivastava

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Laundry detergent is one such indispensable commodity that has been used by consumers all over the world. With the introduction of new and advanced laundry detergents, the global laundry detergent market is expected to rise by 4 percent during the years 2022 -2027. The present paper aims to understand consumer laundry behavior at the household level and how detergents can play a major role in the shedding of microfibers and causing pollution to the environment. Results indicated that water hardness plays a major role in the usage of detergents, but most consumers are ignorant about the dosage of laundry detergent and are using twice or more than the amount of detergent required. This is a potential cause of over usage and pollution, and with the usage of more detergents, more water is required to wash off the residue which otherwise causes skin problems to the users. A heavy dosage of powder detergents can increase the shedding of microfibers up to 193 percent when compared to the liquid detergent. Educating consumers and bringing our suitable legislation may help mitigate the problem to some extent.

Keywords: laundry detergent, microfibers, liquid detergent, powder detergent, environmental effects, domestic laundry.

Classification: DDC Code: 363.738 LCC Code: TD427.D4

Language: English

London Journals Press

LJP Copyright ID: 925672
Print ISSN: 2631-8490
Online ISSN: 2631-8504

London Journal of Research in Science: Natural and Formal

Volume 22 | Issue 7 | Compilation 1.0

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Laundry detergent is one such indispensable commodity that has been used by consumers all over the world. With the introduction of new and advanced laundry detergents, the global laundry detergent market is expected to rise by 4 percent during the years 2022 -2027. The present paper aims to understand consumer laundry behavior at the household level and how detergents can play a major role in the shedding of microfibers and causing pollution to the environment. Results indicated that water hardness plays a major role in the usage of detergents, but most consumers are ignorant about the dosage of laundry detergent and are using twice or more than the amount of detergent required. This is a potential cause of over usage and pollution, and with the usage of more detergents, more water is required to wash off the residue which otherwise causes skin problems to the users. A heavy dosage of powder detergents can increase the shedding of microfibers up to 193 percent when compared to the liquid detergent. Educating consumers and bringing our suitable legislation may help mitigate the problem to some extent.

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I. INTRODUCTION

Laundry is one of the most common and most widespread activities (Pakula & Stamminger, 2010) carried out all around the world (Bianchetti et al., 2015), which is associated with many environmental impacts like the amount of water used, detergent dosage, and water heating (Golsteijn et al., 2015). Huge amounts of laundry detergents with their adjoining components enter the environment on daily basis (Warne & Schifko, 1999). Laundry detergent is mainly subdivided into three main groups; anionic, cationic, and non-ionic. Anionic surfactants are mainly used for soil and dirt removal, cationic surfactants act as a fabric softener and non-ionic surfactants reduce the hardness of water, which in turn helps the anionic surfactants to work their effectiveness on soiled textiles (Cheng et al., 2020).

The global laundry market was expected to show an additional CAGR of 4 percent from 2022 to 2027, with the present market having a value of 62.4 billion USD (for the year 2020) (*Laundry Detergents Market Size, Share, Price Trends, Report 2022-2027*, n.d.).

Numerous brands have been setting up their mark on the industry with several advertisements having keywords like bio-degradable (Batista, 2022), safe for the environment, eco-friendly (Bolt, 2022), low phosphorous, etc. to encourage consumers to purchase their merchandise. Unfortunately, many of these statements fail to reach the expectations or standards of the industry. One pertinent question that remains unanswered is that consumers need to ask about the ingredients in these products and their chemical load. Some laundry detergents with hidden bleaching agents have the potential to kill off the beneficial bacteria present in the waterways (Bianchetti et al., 2015). Studies also suggest that laundry detergents and washing loads can play a major role in the shedding of microfibers from laundered textile items (Volgare et al., 2021). Apart from other concerns consumer knowledge and behavior play a crucial role in the daily basis of following sustainable laundry practices (Kruschwitz et al., 2014).

Thus, a need for the present study arises to suggest the optimization measures which not only control the usage of detergents but also mitigate the microfibers pollution being generated. The main objectives of the present study are to get acquainted with consumers' domestic household laundry practices, consumer attitudes toward the laundry, and how the difference in the usage is responsible for the microfiber generation.

II. METHODOLOGY

A household survey was conducted to find out the laundry practices of the households and to gather information regarding the mode of washing, type of water/ detergent, locality, and different patterns followed during laundry. Additionally, open-ended question-like problems faced during laundry were also added to know about the constraints faced by the respondents. The Snowball sampling technique was used and an online Google survey proforma was developed and circulated among respondents and respondents were encouraged to share the proforma among their circle. A total of 315 responses were obtained, after deleting the duplicate and wrongly filled proforma, 297 responses were finalized for further evaluation.

Laundry effluents samples were collected from the selected households and were analyzed for microfiber contaminants. To optimize the detergent usage and microfibers shedding laundry cycles were run under different quantitative and qualitative measures to get familiar with sheddability triggers.

III. RESULTS

The Snowball sampling method enabled us to get responses from a wider consumer base, Figure 1 depicts the domicile states of the respondents who participated in the study. A total of 297 participants belonging to 15 states shared their opinions. Female respondents were higher (223 responses) than males (74 responses). A striking difference noted is due to the fact that laundry is still considered to be a gender-sensitive role.

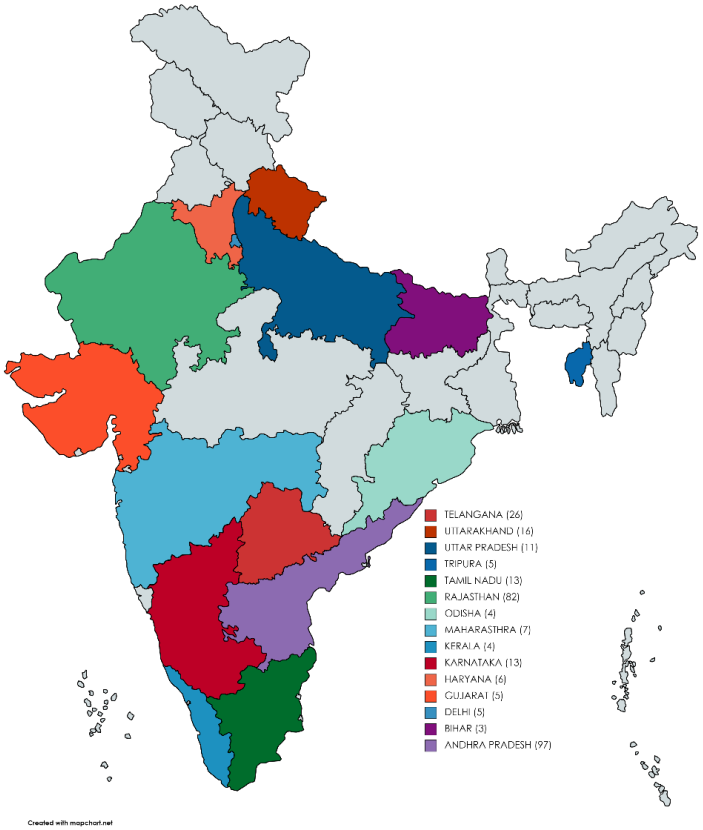

Figure 1: Geographical mapping of the respondents

As age can also play a major role in the understanding of the laundry habits, respondents were categorized to find out that, females and males within the age group of 20- 29 were comparatively more than other categories (Figure 2).

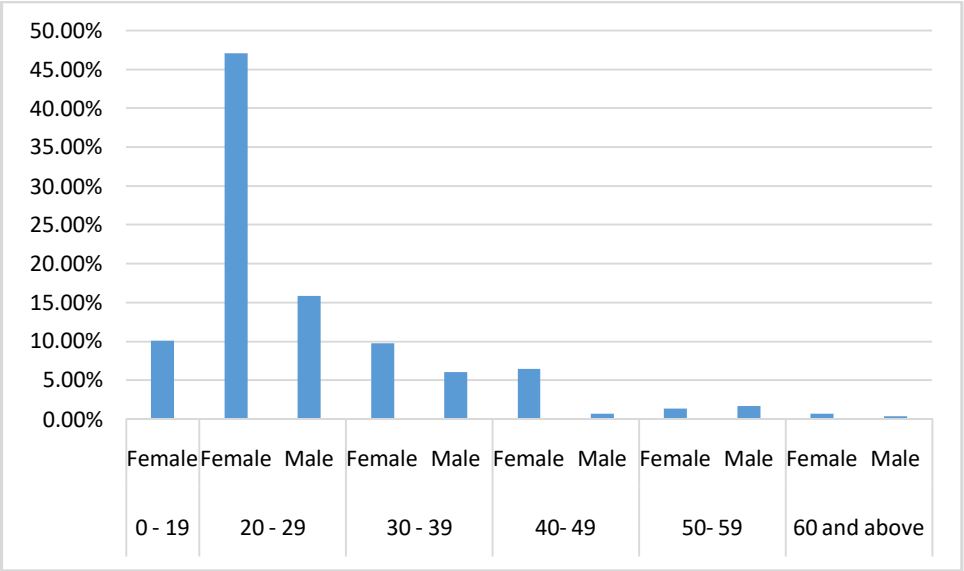

Figure 2: Respondents' age and gender-wise percent

Water has a key role in the laundry process, and water hardness plays a major role in the amount of detergent to be used for the laundry. Table 1 depicts the percentage of the respondents using different water sources and different laundry types. The majority of the respondents (66.33%) were using freshwater, whereas a considerable amount of respondents (29.63 %) were using bore water, and only 0.67 percent of the respondents were using both bore and municipal water. Bore water is considered hard water compared with freshwater. About 1.35 percent of the respondents use saline water to launder their clothes, this type of water requires more detergent than other types of water to make the fabric feel fresh.

Table 1: Percentage of respondents based on the type of water and laundry method

Row Labels	Type of water used for washing
Bore plus municipal water mixed	0.67%
Machine wash, Maid services	0.67%
Bore water	29.63%
Both Hand and Machine wash	12.46%
Both Hand and Machine wash, Dry cleaning	3.03%
Both Hand and Machine wash, Maid services, Dry cleaning	1.35%
Hand wash	5.72%
Machine wash	4.71%
Machine wash, Dry cleaning	0.67%
Machine wash, Maid services, Dry cleaning	1.01%
Maid services	0.67%
Freshwater	66.33%
Both Hand and Machine wash	27.27%
Both Hand and Machine wash, Dry cleaning	5.72%
Both Hand and Machine wash, Maid services	2.02%
Both Hand and Machine wash, Maid services, Dry cleaning	2.02%

Hand wash	9.76%
Hand wash, Dry cleaning	0.67%
Hand wash, Machine wash, Dry cleaning	0.34%
Machine wash	14.14%
Machine wash, Maid services	1.35%
Machine wash, Maid services, Dry cleaning	2.02%
Maid services	1.01%
Lake water	1.68%
Hand wash	1.68%
Saline water	1.35%
Both Hand and Machine wash	0.67%
Both Hand and Machine wash, Maid services	0.34%
Hand wash	0.34%
Well	0.34%
Hand wash	0.34%
Grand Total	100.00%

To get a clear note, Cameron, (2015) in his study observed that liquid detergents were least affected or sometimes not affected by the increase or decrease in water hardness and gave the same results during the washing process. Whereas, powder detergents were most affected and hence necessitated a need for increased usage. The study also gave an insight that powder detergents gave their best performance compared to liquid detergents in favorable/ soft water. Information regarding the type of detergent being was sought from the respondents. A majority of them (56.90 %) opted for the powder detergent, 23.23 percent used liquid, and 16.83 percent used both powder and liquid detergents. Only 3.03 percent of the respondents used eco-friendly or zero waste detergents, which shows the attitude of consumers towards choosing a laundry detergent.

Table 2: Responses concerning the type and brand of the detergent being used (n = 297)

S.No	Parameters	Number of Households*	Percent (%)
1.	Type of detergent being used		
	a) Powder	169	56.90
	b) Liquid	69	23.23
	c) Zerowaste/ Eco-friendly	9	3.03
	d) Both Liquid and Powder	50	16.83
2.	Preferred detergent brands		
	a) Ariel	29	9.76
	b) Ezee	19	6.39
	c) Ghadi	10	3.36
	d) Henko	19	6.39
	e) IFB	9	3.03
	f) Mr White	10	3.36
	g) Praacheen Vidhaan	9	3.03
	h) Rin	59	19.86
	i) Surf excel	118	40.06
	j) Tide	39	13.13
	k) XXX	20	6.73

** Multiple Responses*

The most popular laundry detergent among the subjects was Surf Excel (40.06 %), followed by Rin (19.86 %), Tide (13.13 %), and Ariel (9.76 %). Patterson, (2004) has conducted a study on 40 powder and 21 liquid laundry detergents. Results showed that powder detergents are having a comparatively higher amount of phosphorous content than that of the liquid detergents, and the labeling does not indicate the quantities of these in the detergent, which should be a matter of concern. Indian Standards (BIS) have prescribed the set of standards for the manufacturing of laundry detergents and most of the brands have exceeded the amounts of phosphorous, carbonate, sulfate, etc. IS 4955: 2001 gives three standard grades as per detergents performance (Chaudhary, 2015), and the clause given under the standards suggests that detergent powder should be non-injurious to the fabrics being washed.

Most labels of laundry detergents do not contain the ingredients for a ready reference. This information was provided in a roundabout way with a QR code which has to be scanned by the consumer to know about the ingredients. Most consumers use more laundry detergent than necessary by following the recommended dose on the labels of the detergent, whereas in reality consumers should choose their own dosing practices keeping in mind the requirements of their laundry load.

Figure 3 clearly shows the knowledge gap in the consumer's mind, when enquired about the amount of laundry detergent used, 60.50 percent of the respondents opined that detergent usage is based on the amount of laundry, whereas only 25.00 percent use the detergent based on the amount of uncleanliness/ dirt on the clothing, while 13.90 percent stated that they use the same amount of detergent irrespective of the amount of laundry. It is interesting to note that mainstream responses were found for the statement, based on the amount of laundry, which is not the right practice resulting in excessive usage and waste generation. Instead, consumers should adapt the dosing practices based on the uncleanliness of clothing items. Kruschwitz et al., (2014) studied 236 private households and found out that consumers do not adjust the detergent dosage according to the type of textile, soil level, load size, and water hardness. This leads to over or under dosage of the laundry detergent.

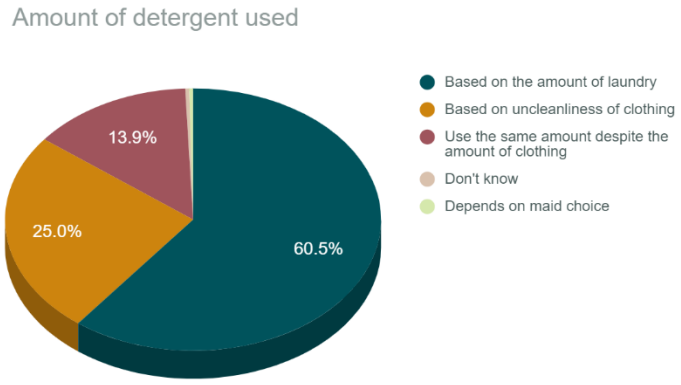

Figure 3: Parameters for the usage of detergent during laundry

Laundry sorting practices have an indirect impact on the shedding of microfibers and the usage of detergents. Synthetic fabrics/ clothing needs a fabric softener to reduce the agitation and abrading to adjacent clothing items while washing, whereas cotton clothing needs considerably a little more detergent to shed off dust and stains. When enquired about the sorting practices, a significant result with a p-value of 0.008631 was found for sorting the laundry by fabric type of content before washing and sorting the laundry by color. Sorting by colour seemed to be the most followed criteria when compared to sorting the laundry by fiber content.

Table 3: Consumer's laundry sorting practices

	Demographic region			P-value	Remark
	Urban	Rural	Semi-urban		
Sort the laundry by fabric type or content before washing	95	51	37	0.008631	Significant
Sort the laundry by fabric color	108	46	50		

(5 % level of significance)

Laundry water from real household conditions was collected without any standardization to get an accurate analysis of the fibers/ microfibers released. Laundered effluents were filtered using a microfiber filter paper with a 0.7 µm pore size. ANOVA showed that there is a significant difference between the detergent types and the number of microfibers released (p=0.001). Figure 4 depicts the statistical box plot depicting that powder detergents were usage have a comparative heightened shedding index than the other two detergent types. In natural detergents, pracheen vidhaan a powder based detergent and soap nuts were used, with soap nuts resulting in less shedding. But the main constrain faced was that soap nuts were not efficient in the removal of hard stains, thus restricting its usage.

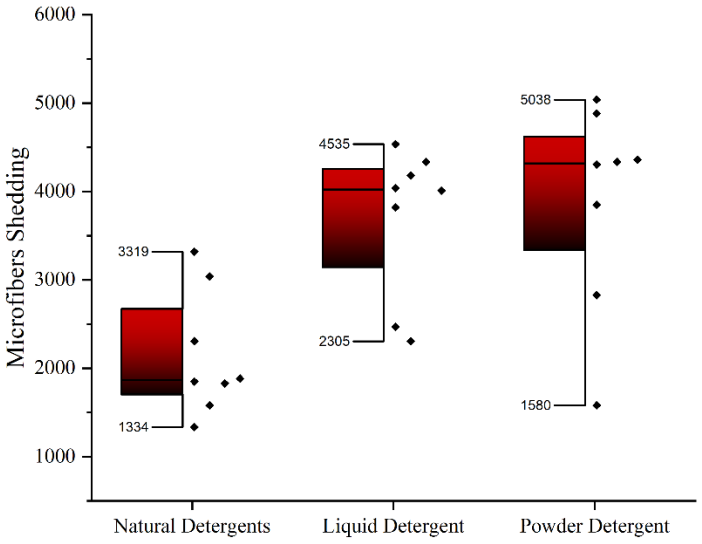

Figure 4: Microfibers shedding rate under different type of laundry detergents Effect of detergent of Microfibers shedding

Similar studies were conducted by researchers stated that detergent usage have an impact on microfibers release. Falco et al., (2017) in a study washed three synthetic textiles namely, polyester, acrylic, and polyester blend, and claimed that usage of powder detergent releases 35330± 664 microfibers per gram of fabric, whereas only 1273 ± 177 microfibers per gram of fabric were released when using a liquid detergent. Hernandez et al., (2017) stated that the usage of detergents (both liquid and powder) is the cause of the release of more microfibers. Napper & Thompson (2016) testified that detergents containing bio-detergent enzymes increased the fiber loss in some washes, but they appeared to decrease or do not have any impact on fiber loss in other washes. Yang et al., (2019) bring in that the usage of detergent has significantly increased the microfiber loss,

especially when polyester/ synthetic fibers are washed at lower temperatures.

SEM Analysis

SEM imaging conducted revealed that (figure 5) laundry effluent have a varied lengths and widths of microfibers, irrespective of the type of detergent used. Apart from laundry detergent, several other factors are responsible for the microfibers shedding, which renders the obtained image inconclusive with respect to the length and width dimesions.

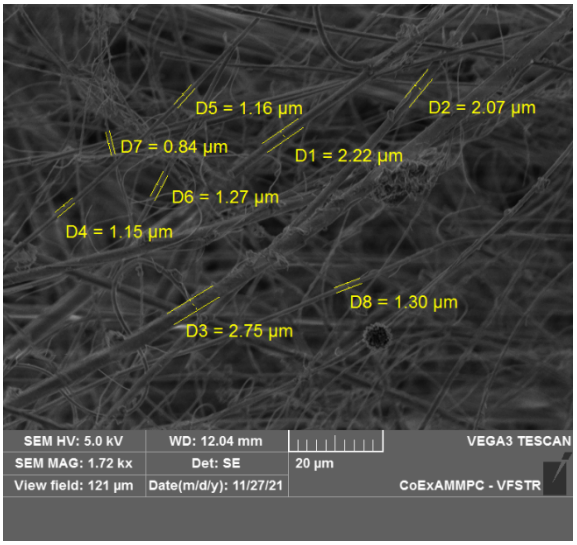

Figure 5: SEM analysis of the laundered effluent showing different fiber diameters

IV. CONCLUSION

To conclude consumer knowledge and laundry practices play a major role in the purchase and utilization of detergents. Powder detergents are more affected by variations in water than liquid detergents. Bleach additives used in the detergents are making the detergent perform well but when released into waterways they are causing harm to aquatic flora and fauna. While educating householders is critical, on-site regulations must be publicized and guidelines and information to be provided on packages. With the increase in microfiber pollution, there is a need to bring out and encourage sustainable laundry practices. Increased levels of awareness and consciousness are the only ways to reduce excessive chemical use in households. This can be a step towards the advancement of effective on-site wastewater treatment systems.

ACKNOWLEDGMENT

I would like to acknowledge Dr. Vijay Lakshmi, Assistant Professor, YSR Horticulture University Thadepalli Gudem, for her guidance in the statistical procedures.

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Keywords: transitivity action, primitivity action, ordered sets of triples, cartesian product and alternating group.

Mathematics Subject Classification: 20B05; 20B15; 20B20

Classification: DDC Code: 330.973 LCC Code: HC106.5

Language: English

London Journals Press

LJP Copyright ID: 925673
Print ISSN: 2631-8490
Online ISSN: 2631-8504

London Journal of Research in Science: Natural and Formal

Volume 22 | Issue 7 | Compilation 1.0

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I. PRELIMINARIES

1.1 Notation and Terminology

In this paper, we shall represent the following notations as: $\sum$- sum over i; A_n -an alternating group of degree n and order $\frac{n!}{2}$; $|G|$ – the order of a group G; $|G:H|$ -Index of H in G;

$P^{[3]}$ – the set of an ordered triple from set $P = \{1, 2, 3,..., n\}$; $S^{[3]}$ – the set of an ordered triple from set $S = \{n + 1, n + 2,..., 2n\}$; $V^{[3]}$ – the set of an ordered triple from set

$V = \{2n + 1, 2n + 2,..., 3n\}$; $[a, b, c]$ -Ordered triple; $A_n \times A_n \times A_n$ -Cartesian product of alternating group A_n; $P^{[3]} \times S^{[3]} \times V^{[3]}$ -Cartesian product of ordered sets of triples $P^{[3]}$, $S^{[3]}$ and $V^{[3]}$.

Definition 1.1.1: Group action (Kinyanjui et al., 2013): Let P be a non-empty set. A group G is said to act on the left of P if for each $g\in G$ and each $p\in P$ there corresponds a unique element $gp\in G$ such that:

(i) $(g_1g_2) = g_1(g_2p)$, $g_1, g_2 \in G$ and $p \in P$.

(ii) For any $p\in P$, $ep = P$, where e is the identity in G.

The action of G from the right on P can be defined in the same manner.

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Definition 1.1.2: Orbit (Njagi, 2016): Let G act on a set P. Then P is partitioned into disjoint equivalent classes called orbits or transitivity classes of the action. For every $p \in P$ the orbit containing p is called the orbit of p and is denoted by $Orb_G(p)$.

Definition 1.1.3 Fixed point (Kinyanjui et al., 2013): Let G act on a set P. The set of elements of P fixed by $g \in G$ is called the fixed-point set of G and is denoted by $Fix(g)$. Thus $Fix(g) = \{gp = p\}$.

Definition 1.1.4: Transitive group (Cameron, 1970): If the action of a group G on set P has only one orbit, then we say that G acts transitively on P. In other words, G acts transitively on P if for every pair of points $p, s \in P$, there exists $g \in G$ such that $gp = s$.

Theorem 1.1.4: (Orbit – Stabilizer Theorem, Rose, 1978, p.72): Let G act on a set P. Then $\left|Orb_G(p)\right| = \left|G: Stab_G(p)\right|$.

Definition 1.1.5: Blocks and primitivity (Nyaga et al., 2011): Assume that the action of G on X is transitive. For every subset A of X such that each $g \in G$, let $gA = \{ga: a \in A\} \subseteq X$. A subset A of X is referred to as a block for the action if, for every $g \in G$, either $gA = A$ or $gA \cap A = \emptyset$. $\emptyset, X$ and all singleton subsets of X are definitely blocks known as trivial blocks. If these are the only blocks, then G acts primitively on X otherwise, G acts imprimitively.

Definition 1.1.6: Direct product action (Cameron et al, 2008): Let (G_1, P_1) and (G_2, P_2) be permutation groups. The direct product $G_1 \times G_2$ acts on the disjoint union $P_1 \cup P_2$ by the rule $p(g_1, g_2) = \{pg_1, \text{ if } p \in P_1,\ pg_2, \text{if } p \in P_2$ and on the Cartesian product $P_1 \times P_2$ by the rule $(p_1, p_2)(g_1, g_2) = (p_1 g_1, p_2 g_2)$.

Theorem 1.1.7: (Armstrong, 2013): The $G_1 \times G_2 \times G_3$-orbit containing $(p, s, v) \in P \times S \times V$ is given by $Orb_{G_1}(p) \times Orb_{G_2}(s) \times Orb_{G_3}(v)$ and the stabilizer of (p, s, v) is given by $Stab_{G_1}(p) \times Stab_{G_2}(s) \times Stab_{G_3}(v)$.

1.2 Introduction

Higman (1964) introduced the rank of a group on finite permutation groups of rank 3. Cameron (1972) worked on the suborbits of multiply transitive permutations and later in 1973 studied the suborbits of primitive groups.

Hamma and Aliyu (2010), on transitivity and primitivity of dihedral groups proved that the dihedral group of degree $2^n (n \geq 2)$ is transitive and primitive. Ndarinyo *et al.,* (2015) showed that the alternating group $A_n = 5, 6, 7$ acts transitively on unordered and ordered triples from the set $P = 1, 2, ..., n$ when $n \leq 7$ through determination of the number of orbits.

Muriuki *et al.,* (2017) showed that for the action of direct product of three symmetric groups on Cartesian product of three sets, the action is both transitive and imprimitive for all $n \geq 2$ and the associated rank is 2^3.

Mutua *et al.,* (2018) showed that the direct product of $S_n \times A_n$, of the symmetric group S_n by the alternating group A_n on the cartesian product $X \times Y$ has its action both transitive and imprimitive when $n \geq 3$. Nyaga (2018) proved that the direct product action of the alternating group on the Cartesian product of three sets is transitive.

Maraka *et al.*, (2021) showed that the action of the cartesian product of the alternating group, $A_n \times A_n \times A_n$, on the cartesian product of $P^{[3]} \times S^{[3]} \times V^{[3]}$, the cartesian product of ordered sets of triples is transitive when $n \geq 5$.

Based on these results we investigate some properties of $A_n \times A_n \times A_n$, the cartesian product action of the alternating group acting on $P^{[3]} \times S^{[3]} \times V^{[3]}$, the cartesian product of ordered sets of triples.

The cartesian product of alternating group $A_n \times A_n \times A_n$, acts on $P^{[3]} \times S^{[3]} \times V^{[3]}$, by the rule;

$$g_1\{([1,2,3],[1,2,4],\ldots,[n,n-1,n-3],[n,n-1,n-2])\} \times g_2\{([n+1,n+2,n+3],[n+1,n+2,n+4],\ldots,[2n,2n-1,2n-3],[2n,2n-1,2n-2])\} \times g_3\{([2n+1,2n+2,2n+3],[2n+1,2n+2,2n+4],\ldots,[3n,3n-1,3n-3],[3n,3n-1,3n-3])\} = \{g_1([1,2,3],[1,2,4],\ldots,[n,n-1,n-3],[n,n-1,n-2]), g_2([n+1,n+2,n+3],[n+1,n+2,n+4],\ldots,[2n,2n-1,2n-3],[2n,2n-1,2n-2]), g_3([2n+1,2n+2,2n+3],[2n+1,2n+2,2n+4],\ldots,[3n,3n-1,3n-3],[3n,3n-1,3n-3])\};$$

$\forall\, g_1, g_2, g_3 \in A_n, \{([1,2,3],[1,2,4],\ldots,[n,n-1,n-3],[n,n-1,n-2])\} \in P^{[3]}$, set of ordered triples from the set $P = \{1,2,3,\ldots,n\}$; $\{([n+1,n+2,n+3],[n+1,n+2,n+4],\ldots,[2n,2n-1,2n-3],[2n,2n-1,2n-2])\} \in S^{[3]}$, set of ordered triples from the set $S = \{n+1,n+2,\ldots,2n\}$;and$\{([2n+1,2n+$

II. MAIN RESULTS

Theorem 2.1: (Maraka et. al., 2021): The action of the cartesian product of the alternating group A_n, $A_n \times A_n \times A_n$, acting on the cartesian product of ordered sets of triples, $P^{[3]} \times S^{[3]} \times V^{[3]}$, is transitive if and only if $n \geq 5$.

Proof: Let $G = G_p \times G_s \times G_v = A_n \times A_n \times A_n$ act on $P^{[3]} \times S^{[3]} \times V^{[3]}$. It suffices to verify that $\left|P^{[3]} \times S^{[3]} \times V^{[3]}\right|$ is equal to $|Orb_G([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3])|$.

Let $|R| = |Stab_G([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3])|$.

So, $(g_p, g_s, g_v) \in G = A_n \times A_n \times A_n$ fixes $([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3]) \in P^{[3]} \times S^{[3]} \times V^{[3]}$ if and only if 1,2 and 3 comes from 1-cycle ofg_p ; $n+1, n+2$ and $n+3$ comes from 1-cycle of$g_s$and $2n+1, 2n+2$ and $2n+3$comes from 1-cycle ofg_v .

Therefore, $|R| = \left|Stab_G([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3])\right|$

$= \left|Stab_{G_p}([1,2,3]) \times Stab_{G_s}([n+1,n+2,n+3]) \times Stab_{G_v}([2n+1,2n+2,2n+3])\right|$

$$|R| = \frac{(n-3)! \times (n-3)! \times (n-3)!}{2 \times 2 \times 2} = \left(\frac{(n-3)!}{2}\right)^3$$

Applying the Orbit-Stabilizer Theorem we get;

$$|Orb_G([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3])|$$

$$= |G : Stab_G([1,2,3],[n+1,n+2,n+3],[2n+1,2n+2,2n+3])|$$

Therefore;

$$|G| = \frac{n! \times n! \times n!}{2 \times 2 \times 2} = \left(\frac{n!}{2}\right)^3$$

$$\frac{|G|}{|R|} = \left(\frac{n!}{(n-3)!}\right)^3 = \left|P^{[3]} \times S^{[3]} \times V^{[3]}\right|$$

Hence, $A_n \times A_n \times A_n$ acts transitively on $P^{[3]} \times S^{[3]} \times V^{[3]}$ if $n \geq 5$.

Lemma 2.2: The action of the cartesian product of the alternating group A_5, $A_5 \times A_5 \times A_5$, acting on the cartesian product of ordered sets of triples, $P^{[3]} \times S^{[3]} \times V^{[3]}$, is imprimitive.

Proof: This action is transitive by Theorem 2.1.

Now, for $n = 5$, the set $P = \{1, 2, 3, 4, 5\}$, so, $gap > Arrangements([1, 2, 3, 4, 5], 3)$;

$P^{[3]} = \{[\,1,2,3\,],[\,1,2,4\,],\ldots,[\,5,4,1\,],[\,5,4,2\,],[\,5,4,3\,]\}$;

$S = \{6,7,8,9,10\}$, gap> Arrangements([6,7,8,9,10],3);

$S^{[3]} = \{[\,6,7,8\,],[\,6,7,9\,],[\,6,7,10\,],\ldots,[\,10,9,6\,],[\,10,9,7\,],[\,10,9,8\,]\}$and

$V = \{11,12,13,14,15\}$, so gap> Arrangements([11,12,13,14,15],3);

$V^{[3]} = \{[\,11,12,13\,],[\,11,12,14\,],\ldots,[\,15,14,11\,],[\,15,14,12\,],[\,15,14,13]\}$.

We have;

$K =$
$\{([1,2,3],[1,2,4],[1,2,5],\ldots,[5,4,1],[5,4,2],[5,4,3]) \times$
$([6,7,8],[6,7,9],[6,7,10],\ldots,[10,9,6],[10,9,7],[10,9,8]) \times$
$([11,12,13],[11,12,14],[11,12,15],\ldots,[15,14,11],[15,14,12],[15,14,13])\}$

Let K' be the non-trivial subset of $K, K' = P^{[3]'} \times S^{[3]'} \times V^{[3]'}$ such that $|K'|$ divides $|K|$.

So, we have; $\frac{\overline{(5-3)!} \times \overline{(5-3)!} \times \overline{(5-3)!}}{\frac{5!}{\overline{(5-3)!}}} = \frac{|K|}{|K'|}$

Now,

$K' =$

$\{([1,2,3],[6,7,8],[11,12,14]),\ldots,([1,2,3],[6,7,8],[15,14,13]),([1,2,3],[6,7,9],[11,12,13])\}$

therefore, $|K'| = \frac{5!}{(5-3)!}$.

For each element of K' there exist $(g_p, g_s, g_v)\epsilon G$ with $\frac{5!}{(5-3)!}$ cycles permutation,

$\{([1,2,3],\ldots,[5,4,3]),([6,7,8],,\ldots,[10,9,8]),([11,12,13],\ldots,[15,14,13])\}$ such

that for every $(g_p, g_s, g_v)\epsilon G$; $[1, 2, 3]$ is fixed in g_p , $[6, 7, 8]$ is fixed in g_s and that $[11, 12, 13]$ belongs to a single cycle of g_v , then, (g_p, g_s, g_v) either fixes an element of $K^{'} = P^{[3]^{'}} \times S^{[3]^{'}} \times V^{[3]}$ or takes one element of $K^{'}$ to another so that; $(g_p, g_s, g_v)K^{'} = K^{'}$. Any other $(g_p, g_s, g_v)\epsilon G$ moves an element of $K^{'}$ to an element not in $K^{'}$ so that; $(g_p g_s, g_v)K^{'} \cap K^{'} = \emptyset$. Thus, $K^{'}$ is a non-trivial block for the action and it follows from definition 1.1.5 that the action is imprimitive.

Lemma 2.3: The action of the cartesian product of the alternating group A_6, $A_6 \times A_6 \times A_6$, acting on the cartesian product of ordered sets of triples, $P^{[3]} \times S^{[3]} \times V^{[3]}$, is imprimitive.

Proof: This action is transitive by Theorem 2.1.

Now, for $n = 6$, the set $P = \{1, 2, 3, 4, 5, 6\}$, so, gap> Arrangements([1,2,3,4,5,6],3);

$P^{[3]} = \{[1,2,3],[1,2,4],[1,2,5],\ldots,[6,5,2],[6,5,3],[6,5,4]\}$;

$S = \{7,8,9,10,11,12\}$, so gap> Arrangements([7,8,9,10,11,12],3);

$S^{[3]} = \{[7,8,9],[7,8,10],[7,8,11],\ldots,[12,11,8],[12,11,9],[12,11,10]\}$ and

$V = \{13,14,15,16,17,18\}$, so gap> Arrangements([13,14,15,16,17,18],3);

$V^{[3]} =$

$\{[13,14,15],[13,14,16],[13,14,17],\ldots,[18,17,14],[18,17,15],[18,17,16]\}$.

We have;

$K =$

$\{([1,2,3],[1,2,4],[1,2,5],\ldots,[6,5,2],[6,5,3],[6,5,4]) \times$

$([7,8,9],[7,8,10],[7,8,11],\ldots,[12,11,8],[12,11,9],[12,11,10]) \times$

$([13,14,15],[13,14,16],[13,14,17],\ldots,[18,17,14],[18,17,15],[18,17,16])\}$

Let K' be the non-trivial subset of $K, K' = P^{[3]'} \times S^{[3]'} \times V^{[3]'}$ such that $|K'|$ divides $|K|$.

We have; $\frac{\frac{6!}{(6-3)!} \times \frac{6!}{(6-3)!} \times \frac{6!}{(6-3)!}}{\frac{6!}{(6-3)!}} = \frac{|K|}{|K'|}$

Now,

$K' =$

$\{([1,2,3]\ ,[\ 7,8,9],[13,14,16]),\ldots,([1,2,3]\ ,[7,8,9\ ],[\ 18,17,16]),([1,2,3]\ ,[7,8,10\ ],[13,14,15])\}$

therefore, $|K'| = \frac{6!}{(6-3)!}$.

For each element of $K^{'}$ there exist $(g_p, g_s, g_v) \epsilon G$ with $\frac{6!}{(6-3)!}$ cycles permutation, $\{([1,\ 2,\ 3\ ],\ldots,[\ [\ 6,\ 5,\ 4\ ]),([7,\ 8,\ 9\ ],,\ \ldots,\ [12,\ 11,\ 10\ ]),([13,\ 14,\ 15\ ],\ldots,[18,\ 17,\ 16])\}$ such that for every $(g_p, g_s, g_v) \epsilon G$; $[\ 1,\ 2,\ 3\ ]$ is fixed in g_p , $[\ 7,\ 8,\ 9\ ]$ is fixed in g_s and that $[\ 13,\ 14,\ 15\ ]$ belongs to a single cycle of g_v , then, (g_p, g_s, g_v) either fixes an element of $K^{'} = P^{[3]^{'}} \times S^{[3]^{'}} \times V^{[3]^{'}}$ or takes one element of $K^{'}$ to another so that; $(g_p, g_s, g_v)K^{'} = K^{'}$. Any other $(g_p, g_s, g_v) \epsilon G$ moves an element of $K^{'}$ to an element not in $K^{'}$ so that; $(g_p, g_s, g_v)\ K^{'} \cap K^{'} = \emptyset$. Thus, $K^{'}$ is a non-trivial block for the action and it follows from definition 1.1.5 that the action is imprimitive.

Lemma 2.4: The action of the cartesian product of the alternating group A_7, $A_7 \times A_7 \times A_7$, acting on the cartesian product of ordered sets of triples, $P^{[3]} \times S^{[3]} \times V^{[3]}$, is imprimitive.

Proof: This action is transitive by Theorem 2.1.

Now, for $n = 7$, the set $P = \{1, 2, 3, 4, 5, 6, 7\}$, so, gap> Arrangements ([1,2,3,4,5,6,7],3);

$P^{[3]} = \{[1, 2, 3\ ], [\ 1, 2, 4\ ], [\ 1, 2, 5\ ], \ldots, [\ 7, 6, 3\ ], [\ 7, 6, 4\ ], [\ 7, 6, 5\ ]\}$;

$S = \{8,9,10,11,12,13,14\}$, sogap> Arrangements([8,9,10,11,12,13,14],3);

$S^{[3]} = \{[8, 9, 10\ ], [\ 8, 9, 11\ ], [\ 8, 9, 12\ ], \ldots, [\ 14, 13, 10\ ], [\ 14, 13, 11\ ], [\ 14, 13, 12\ ]\}$ and

$V = \{15,16,17,18,19,20,21\}$, sogap> Arrangements([15,16,17,18,19,20,21],3); $V^{[3]} =$

$\{[\ 15, 16, 17\ ], [\ 15, 16, 18\ ], [\ 15, 16, 19], \ldots, [\ 21, 20, 17\ ], [\ 21, 20, 18\ ], [\ 21, 20, 19\ ]\}$.

We have;

$K =$

$\{([1, 2, 3\ ], [\ 1, 2, 4\ ], [\ 1, 2, 5\ ], \ldots, [\ 7, 6, 3\ ], [\ 7, 6, 4\ ], [\ 7, 6, 5\ ]) \times$

$([8, 9, 10\ ], [\ 8, 9, 11\ ], [\ 8, 9, 12\ ], \ldots, [\ 14, 13, 10\ ], [\ 14, 13, 11\ ], [\ 14, 13, 12\ ]) \times$

$([\ 15, 16, 17\ ], [\ 15, 16, 18\ ], [\ 15, 16, 19], \ldots, [\ 21, 20, 17\ ], [\ 21, 20, 18\ ], [\ 21, 20, 19\ ])\}$

Let K' be the non-trivial subset of $K, K' = P^{[3]'} \times S^{[3]'} \times V^{[3]'}$ such that $|K'|$ divides $|K|$

so, we have; $\frac{\frac{(7-3)!}{7!} \times \frac{(7-3)!}{7!} \times \frac{(7s-3)!}{7!}}{\frac{7!}{(7-3)!}} = \frac{|K|}{|K'|}$

.

$K' =$

$\{([1,2,3]\ ,[8,9,10],[15,16,18]),\ldots,([1,2,3]\ ,[8,9,10\ ],[\ 21,20,19\ ]),([1,2,3]\ ,[8,9,11],[15,16,17])\}$

therefore, $|K'| = \frac{7!}{(7-3)!}$.

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For each element of $K^{'}$ there exist $(g_p, g_s, g_v) \epsilon G$ with $\frac{7!}{(7-3)!}$ cycles permutation, {([1, 2, 3],..., [7, 6, 5]), ([8, 9, 10],, ..., [14, 13, 12]), ([15, 16, 17],..., [21, 20, 19])} such that for every $(g_p, g_s, g_v) \epsilon G$; [1, 2, 3] is fixed in g_p , [8, 9, 10] is fixed in g_s and that [15, 16, 17] belongs to a single cycle of g_v , then, (g_p, g_s, g_v) either fixes an element of $K^{'} = P^{[3]^{'}} \times S^{[3]^{'}} \times V^{[3]^{'}}$ or takes one element of $K^{'}$ to another so that; $(g_p, g_s, g_v)K^{'} = K^{'}$. Any other $(g_p, g_s, g_v) \epsilon G$ moves an element of $K^{'}$ to an element not in $K^{'}$ so that; $(g_p, g_s, g_v)\, K^{'} \cap K^{'} = \emptyset$. Thus, $K^{'}$ is a non-trivial block for the action and it follows from definition 1.1.5 that the action is imprimitive.

Theorem 2.5: The action of the cartesian product of the alternating group A_n, $A_n \times A_n \times A_n$, acting on the cartesian product of ordered sets of triples, $P^{[3]} \times S^{[3]} \times V^{[3]}$, is imprimitive for $n \geq 5$.

Proof: The action of $A_n \times A_n \times A_n$ on $P^{[3]} \times S^{[3]} \times V^{[3]}$ is transitive by Theorem 2.1 for $n \geq 5$.

Consider, $\forall\, g_1, g_2, g_3 \in A_n, \{([1,2,3], [1,2,4], \ldots, [n, n-1, n-3], [n, n-1, n-2])\} \in P^{[3]}$, set of ordered triples from the set $P = \{1,2,3, \ldots, n\}$; $\{([n+1, n+2, n+3], [n+1, n+2, n+4], \ldots, [2n, 2n-1, 2n-3], [2n, 2n-1, 2n-2\,])\} \in S^{[3]}$,

set of ordered triples from the set

$S = \{n+1, n+2, \ldots, 2n\}$; and$\{([2n+1, 2n+2, 2n+3], [2n+1, 2n+2, 2n+4], \ldots, [3n, 3n-1, 3n-3], [3n, 3n-1, 3n-3])\} \in V^{[3]}$,

set of ordered triples from the set $V = \{2n+1, 2n+2, \ldots, 3n\}$.

We have;

$$K = \{([1,2,3], [1,2,4], \ldots, [n, n-1, n-3], [n, n-1, n-2]) \times ([n+1, n+2, n+3], [n+1, n+2, n+4], \ldots, [2n, 2n-1, 2n-3], [2n, 2n-1, 2n-2]) \times ([2n+1, 2n+2, 2n+3], [2n+1, 2n+2, 2n+4], \ldots, [3n, 3n-1, 3n-3], [3n, 3n-1, 3n-3])\}.$$

Let $K^{'}$ be the non-trivial subset of K; $K^{'} = P^{[3]^{'}} \times S^{[3]^{'}} \times V^{[3]^{'}}$ such that $|K^{'}|$ divides $|K|$.

Therefore; $$\frac{\frac{n!}{(n-3)!} \times \frac{n!}{(n-3)!} \times \frac{n!}{(n-3)!}}{\frac{n!}{(n-3)!}} = \frac{|K|}{|K'|}$$

$$\text{Now, } K' = \begin{Bmatrix} ([1,2,3], [n+1, n+2, n+3], [2n+1, 2n+2, 2n+4]), \ldots, \\ ([1,2,3], [n+1, n+2, n+3], [3n, 3n-1, 3n-3]), \\ ([1,2,3], [n+1, n+2, n+4], [2n+1, 2n+2, 2n+3]) \end{Bmatrix}.$$

So, $|K'| = \frac{n!}{(n-3)!}$

London Journal of Research in Science: Natural and Formal

For every element of $K^{'}$ there exist $(g_p, g_s, g_v)\epsilon G$ with $\frac{n!}{(n\ 3)!}$ cycles permutation;

$\{([1,2,3],[1,2,4],\ldots,[n,n-1,n-3],[n,n-1,n-2]),([n+1,n+2,n+3],[n+1,n+2,n+4],\ldots,[2n,2n-1,2n-3],[2n,2n-1,2n-2]),([2n+1,2n+2,2n+3],[2n+1,2n+2,2n+4],\ldots,[3n,3n-1,3n-3],[3n,3n-1,3n-3])\}$ such that for every $(g_p, g_s, g_v)\epsilon G$; $[1,2,3]$ is fixed in g_p, $[n+1,n+2,n+3]$ is fixed in g_s and that $[2n+1,2n+2,2n+3]$ belongs to a single cycle of g_v, then, (g_p, g_s, g_v) either fixes an element of $K' = P^{[3]'}s \times S^{[3]'} \times V^{[3]'}$ or takes one element of K' to another so that; $(g_p, g_s, g_v)P^{[3]'} \times S^{[3]'} \times V^{[3]'} = P^{[3]'} \times S^{[3]'} \times V^{[3]'}$. Any other $(g_p, g_s, g_v)\epsilon G$ moves an element of K' to an element not in K' so that; $(g_p, g_s, g_v)P^{[3]'} \times S^{[3]'} \times V^{[3]'} \cap P^{[3]'} \times S^{[3]} \times V^{[3]} = \emptyset$. This argument shows that K is a non-trivial block for the action and the conclusion follows from definition 1.1.5 hence the action is imprimitive for $n\geq 5$.

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An Empirical Investigation on the Scope of Genetic Improvement of *Lavandula angustifolia* Mill. using Somaclonal Variation in the Context of Indian Agro-Climatic Environment

Sougata Sarkar & Deepak Sharma

ABSTRACT

Traditional cultivation of lavender occurs through vegetative means in mostly all lavender growing counties of the world. As a result genetic variation in the germplasm is negligible, which offers serious limitations in classical breeding programs of lavender. This led us to explore the potential of plant tissue culture methods (developing somaclonal variants followed by the selection of putative variants) for inducing genetic variation which would eventually serve the purpose of genetic improvement in lavender. Induction of heritable genetic variations in agro-economic traits through in-vitro micro propagation techniques is the main aim of this study.

Sterile cultures of lavender were produced. The regular phases of somaclonal plantlet development i.e. callogenesis followed by caulogenesis, rhizogenesis was modified by an intervening cell suspension culture phase represented by: callogenesis-1 (calli-1 derived from organised structures i.e. explants) followed by the intervening cell suspension culture (derived from calli-1), callogenesis-2 (calli-2 derived from cell suspension culture), caulogenesis (from calli-2), and finally rhizogenesis.

Keywords: correlation; genetic improvement; hs-gcms; *lavandula angustifolia*; post-hoc tests; somaclonal variation.

Classification: DDC Code: 576.54 LCC Code: QH401

Language: English

London Journals Press

LJP Copyright ID: 925674
Print ISSN: 2631-8490
Online ISSN: 2631-8504

London Journal of Research in Science: Natural and Formal

Volume 22 | Issue 7 | Compilation 1.0

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Traditional cultivation of lavender occurs through vegetative means in mostly all lavender growing counties of the world. As a result genetic variation in the germplasm is negligible, which offers serious limitations in classical breeding programs of lavender. This led us to explore the potential of plant tissue culture methods (developing somaclonal variants followed by the selection of putative variants) for inducing genetic variation which would eventually serve the purpose of genetic improvement in lavender. Induction of heritable genetic variations in agro-economic traits through in-vitro micro propagation techniques is the main aim of this study.

Sterile cultures of lavender were produced. The regular phases of somaclonal plantlet development i.e. callogenesis followed by caulogenesis, rhizogenesis was modified by an intervening cell suspension culture phase represented by: callogenesis-1 (calli-1 derived from organised structures i.e. explants) followed by the intervening cell suspension culture (derived from calli-1), callogenesis-2 (calli-2 derived from cell suspension culture), caulogenesis (from calli-2), and finally rhizogenesis. The aspect of quantifiability was incorporated by the modified approach. Twenty somaclones exhibited significant differences in twenty one traits reflecting the induction of somaclonal variation within them. Somaclones 5, 6, and 10 among twenty somaclonal variants were genetically improved promising candidates.

Keywords: correlation; genetic improvement; hs-gcms; *lavandula angustifolia*; post-hoc tests; somaclonal variation.

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I. INTRODUCTION

Lavandula officinalis Chaix. synonym *L. angustifolia* Mill. (lavender) is a perennial, branched, bushy shrub belonging to the family Lamiaceae. Lavender is one of the most prestigious cash crop in the world, grown for its expensive essential oil having use in medicine (therapeutic effects as anti-anxiety disorders, sedative, spasmolytic, antiviral , and antibacterial agent), food (as a natural flavouring for beverages, ice cream, sweets, baked goods , and chewing gum), perfumery (cosmetics), in aroma-therapy (as a relaxant) (Da Porto et al. 2009) , and a steady agro-industrial business has come up with this aromatic crop during the last few decades (Lis-Balchin 2002). Amongst all other species of *Lavandula,* only three species are of industrial importance e.g. *Lavandula angustifolia*, *Lavandula latifolia* and *Lavandula hybrida* (*Lavandula latifolia* × *Lavandula angustifolia*), which produce lavender oil, spike lavender oil, and lavandin oil respectively (Lis-Balchin 2002; Lubbe and Verpoorte 2011).

Colonial (British) India suffered from abortive attempts for commercial cultivation of lavender. The history of lavender cultivation in independent India dates back to the year 1957, when Sir Col. R.

N. Chopra introduced this plant in the Kashmir valley on a very small scale due to which commercial exploitation could not be possible. Changing political scenario in the province of Jammu and Kashmir also accounted for the failure of his efforts (Singh et al. 2007). It was after the systematic intervention of CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow during early 80's, that about 100 hectares of land was brought under lavender cultivation in Kashmir along with development of appropriate agro-technologies. Essential oil obtained from steam distillation of flowering spikes produced at Pulwama farm (presently situated in the Union territory of Kashmir) since then met the international standards and are still being traded to the perfumery industry (Verma et al. 2010; Shawl et al. 2000; Handa et al. 1957). Apart from the successful introduction and sustainable cultivation of lavender in Jammu and Kashmir, CSIR-Institute of Himalayan Bio resource Technology (IHBT), Palampur during year 2000, had also been successful to introduce and cultivate lavender on a semi-commercial scale covering the district of Chamba in Himachal Pradesh (Singh et al. 2007). In the recent past, much of the efforts to popularise lavender cultivation in newer pockets in the Union Territories of Kashmir and Jammu was undertaken by CSIR-Indian Institute of Integrative Medicine (IIIM). In the light of the plant's habit and habitat, cultivation and extension programs, lavender has been restricted to higher altitudes (The Himalayan landscape) in India.

The aerial parts of perennial bushy lavender consists of an erect woody stem, its branches, leaves that are simple, entire, opposite, lanceolate, aromatic, greyish-green in colour with slight hairy appearance. Every branch terminates in a spike packed with small violet aromatic flowers. For luxuriant growth and development of lavender, a well-drained soil system such as sandy, sandy loam or gravelly soil is a prerequisite with its *pH* between 6.5 to 7.5. Lavender, in India is traditionally propagated by softwood cuttings. Propagation through hardwood cuttings are generally avoided due to delayed root initiation. In India, flowers of lavender bloom mainly in the valley of Kashmir along with some scattered pockets in Jammu having similar agro-climatic conditions. Hence, vegetative methods of propagation is popular, favoured and relied (for raising uniform population of genotypes, quality and quantity of essential oil yield) over seed propagation methods (as genetic uniformity is compromised in the progeny populations) by the growers across this province.

Genetic improvement of lavender through classical plant breeding approach was initiated in India by CIMAP Regional Centre, Kashmir in 1978. Polycross mating design was implemented to achieve maximum diversity among progeny. By the end of 1988, the Centre was credited to develop forty three genotypes of lavender comprising twenty parental lines and twenty three clonal lines. As a result, the variety 'Karlovo' was released as an outcome of introduction and a clone, now known as 'Sher-i-Kashmir' came into existence (Singh et al. 1989). Since then, nothing much significant was done with the aspect of genetic improvement in lavender and the last or may be the only well recognized variety called 'Sher-i-Kashmir' was developed more than three decades ago from Indian perspective. However, genetic improvement programs continued in other scientific communities of the world who improvised and adapted classical plant breeding approaches (Hassiotis et al. 2010), polyploid induction model (Urwin et al. 2007; Urwin, 2009; Urwin et al. 2014), mutation breeding approach (Badawy et al. 2003), transgenics (Muñoz-Bertomeu et al. 2006; Ibrahim et al. 2017; Landmann et al. 2007; Mendoza-Poudereux et al. 2014) and plant tissue culture (PTC) techniques (Tsuro et al. 2009; Keykha et al. 2014; Andrys and Kulpa 2018; Onisei et al. 1994; Onisei et al. 1999) to induce genetic variation in the existing lavender germplasm.

PTC techniques are reported in some *Lavandula* sp. e.g., axillary shoot proliferation was reported for *Lavandula dentata* (Jordan et al. 1998; Sudria et al. 1999, 2001; Echeverrigaray et al. 2005), *Lavandula latifolia* (Sanchez-Gras and Calvo 1996) and *Lavandula vera* (Andrade et al. 1999). Direct regeneration of shoots from different

explants was reported for *L. latifolia* (Calvo and Segura 1989a) and indirect regeneration of shoots (having an intervening callus phase) was reported in *Lavandula angustifolia* (Quazi 1980; Ghiorghita et al. 2009), *Lavandula* × *intermedia* (Dronne et al. 1999), *L. latifolia* (Calvo and Segura 1988, 1989b; Jordan et al. 1990), *Lavandula officinalis* × *L. latifolia* (Panizza and Tognoni 1988) and *L. vera* (Tsuro et al. 1999, 2000).

In order to initiate any genetic improvement program, the minimum prerequisite is to have an optimum genetic variation in the existing germplasm of the specified crop. The maximum exploitation of clonal propagation method, for decades, in the context of lavender cultivation in India has led to narrowing its genepool. Considering all the above aspects, it is highly imperative that genetic improvement leading to varietal development in lavender will be a very difficult task through classical breeding approach unless the magnitude of variation in the lavender population rises to an optimal level. Occurrence of variation in nature is very slow hence, induced variation is the only favourable and novel option in this regard. In the light of all these above aspects, application of somaclonal variation technique through plant tissue culture (PTC) approach to induce variability among indirectly regenerated and *in-vitro* raised lavender plantlets were considered as a novel approach in this present study for bridging the gap of genetic improvement endeavours in Indian perspective. A schematic representation of the entire endeavour is reflected in the figure (1).

II. MATERIALS AND METHODS

2.1 Plant material and establishment of sterile in-vitro cultures

Healthy apical shoot meristems were excised from lavender plants growing in glass house condition for establishing aseptic cultures. These explants were surface sterilized with Tween-20, followed by 70% ethanol wash for 30s and finally with 0.10% $HgCl_2$ treatment for 45 s followed by five washings with sterile double distilled water before implanting onto a modified Murashige and Skoog, 1962 basal medium (MS_0) supplemented with 0.20 ppm thiamine-HCl, 1.00 ppm pyridoxine-HCl, 4.00 ppm glycine, 100.00 ppm myo-inositol, 0.70% agar and 3.00% sucrose. The pH of the media was adjusted to 5.80 prior autoclaving at 121.00°C and 117.70 kPa for 15 min. All aseptic cultures were incubated at 25±2°C with a photoperiod of 16 h under fluorescent light (40 to 50 µmol $m^{-1}s^{-1}$).

2.2 Classification of culture medium

The nodes, internodes, apical shoots, and leaves were used as explants from axenic *in-vitro* plantlets. The $0.5MS_0$(half strength of MS_0) and MS_0 supplemented with (1.00, 3.00, 5.00 ppm) 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with (0.25, 0.50 ppm) Kinetin (Kin) and $0.5MS_0$ and MS_0 supplemented with (0.50, 1.00 ppm) Indole-3-acetic acid (IAA) in combination with (0.50, 1.00 ppm) Indole-3-butyric acid (IBA) were examined for selection of effective callus inducing medium(s) from forty media combinations mentioned above.

A portion of the callus (weighing 1.00 g) was subcultured into 100.00 ml liquid MS_0 (without agar) medium and incubated on a rotary shaker with constant agitation (70 rpm) in the dark until individual cells of the callus could be observed as a uniform suspension in the liquid medium. From this suspension culture, 20 µl was transferred onto the most effective callus inducing medium screened from the above medium combinations. The remaining callus were repeatedly subcultured onto fresh callus propagation medium.

Calli subcultured onto $0.5MS_0$, MS_0, and MS_0 supplemented with (1.00, 2.00 ppm) 6-benzylamino purine (BAP), MS_0 supplemented (0.25, 0.50 ppm) Kinetin (Kin) alone and in combination were examined for selection of effective shoot inducing medium(s) from ten media combinations mentioned above.

The healthy adventitious shoots (5-7 cm long) obtained upon indirect regeneration were excised from the culture. These shoots were subcultured onto MS_0, $0.5MS_0$, and $0.5MS_0$ supplemented with 0.50 ppm Indole-3-butyric acid (IBA) and were examined for selection of effective root inducing

medium(s) from three media combinations mentioned above.

On the other hand, cuttings of apical shoot meristems from a donor lavender plant growing in open field condition were subjected to rooting in (1:2) sand-soil mixture which will subsequently act as checks/controls.

The most healthy and complete *in-vitro* plantlets derived through caulogenesis were selected for acclimatization and were gradually shifted to glasshouse condition for further hardening and evaluation of somaclonal variations.

2.3 Acclimatization and hardening

Twentynine acclimatized somaclones (SC) along with two checks were initially transplanted into small pots containing 2:1:1:1 mixture of soil: sand: vermiculite: farmyard manure and hardened in the glasshouse of the experimental farm of CSIR-IIIM, located at Chatha, Jammu, INDIA. These complete plantlets were irrigated with 0.5MS_0 liquid medium without sucrose as and when required during initial days of glasshouse establishment. Later it was entirely replaced by tap water. Twenty regenerated plantlets with two checks finally attained proper vegetative growth and development and were transferred to bigger pots with precautions for minimum root damage. Neither the somaclones nor the checks bloomed post glasshouse transfer.

2.4 Selection of specific traits for estimation of somaclonal variation.

Data of six morphometric traits (Figure 2) like plant height (height of the tallest branch of the potted plant from the pot soil surface measured in centimetres), number of branches (total number of lateral branches emanating from the main/primary stem), fresh weight of plant (total weight of the shoot and root system of the live plant in grams), root length (measure of the total root length in centimetres), stem circumference (circular measurement of the stem just above the pot soil of the potted plant measured in centimetres), branch length (length of a branch from the apical tip to its base on the primary axis measured in centimetres) and fifteen major essential oil components like - eucalyptol; endo-Borneol; 3-Carene; camphene; α-Pinene; p-Cymene; o-Cymene; cyclobutane,1,2-dicy-clopropyl-; bicycle [2.2.1] heptan- 2-ol,1,7,7-trimethyl-, (1S-endo)-; linalool; D-Limonene; (+)-2-Bornanone; α-Phellandrene; β-Phellandrene; linalyl acetate; extracted from sampled leaves using HS-GCMS for twenty somaclones with two checks (i.e. 22 treatments) growing in bigger pots in glasshouse condition were recorded.

2.5 Head Space-Gas Chromatography-Mass Spectrometry (HS-GCMS)

The qualitative and quantitative estimation of leaf volatiles for glass house grown treatments were performed on a Shimadzu Nexis GC-2030 hyphenated with GCMS-TQ8040 instrument and samples were introduced through HS-20 headspace sampler. Fresh leaf material of 500 mg sample was kept in 20 ml head space flat base vial fitted with crimp cap and silicon/PTFE 18 mm 35 SHORE septum. The vial was incubated in head space heater for 5 min at 120°C with 50 kps pressure. The loop temperature was 110°C and transfer-line temperature was kept at 120°C. The sample was introduced into the split/splitless injector in the split mode (1:25) at 280°C. The column oven temperature was programmed from 90°C to 120°C at the rate of 3°C/min with final hold of 2 min. High purity helium gas was used as a carrier gas (1 ml/min) and a SH-Rxi-5S MS (30 m × 0.25 mm; 0.25 µm film thickness) column was employed for separation. Identification of compounds was based on retention time, elution order, relative retention index using a homologous series of n-alkanes (C_8–C_{25} hydrocarbons) with those of literature. Further identification was made by matching the recorded mass spectra with those stored in the inbuilt mass spectral library. The percentage determination was based on peak area normalization.

2.7 Experimental design and data analysis

The experiments were conducted using a completely randomized design. The statistical analysis was performed by one-way analysis of variance (ANOVA). Tukey-Kramer's, Scheffe's and Student's T (Bonferoni corrected) post hoc tests,

($p \leq 0.05$) was performed using SPSS statistical software (version 20 for Windows) and MS Excel-2007. Correlation coefficients were calculated amongst all traits and significant positive and negative values were demarcated. The regression equation, $\hat{Y} = c + m_i x_i$, where, '$\hat{Y}$' represents dependent trait, 'x' represents independent traits, 'm' represented slope of x, 'i' represents number of traits and 'c' represents intercept on $\hat{Y}$ axis was formulated. SET Theory was also involved to demonstrate and simplify the comparison of efficacy among three post hoc tests.

III. RESULTS

3.1 Establishment of in-vitro cultures

The apical shoot meristems of *Lavandula angustifolia* Mill. (lavender) growing in glasshouse condition was used as explants for sterile *in-vitro* culture establishment. Almost 75% of the explants completely recovered from the inevitable surface sterilization stress within two weeks of inoculation. These explants exhibited quick growth response in MS_0. The lateral adventitious shoots emanating from them were subcultured onto shoot proliferation mediums to produce a stock of axenic plantlets which were utilized for all upcoming experiments. MS_0 supplemented with 1.00 ppm BAP and 0.50 ppm Kin exhibited best growth response while $0.5MS_0$ exhibited least growth response to adventitious shoot proliferation in lavender.

3.2 Callogenesis

The nodes, internodes, leaves, and shoot apices of *in-vitro* established lavender plantlets were subcultured onto callus inducing mediums. Within a span of two weeks, although every explant responded to initial callus induction, however the best response was observed in apical shoots followed by leaves, nodes, and internodes. However, in the end of fifth week, internodal explants giving rise to inconspicuous mass of callus, ceased to exist. On the other hand, nodal and leaf explants gave rise to friable callus but maximum proliferation of the same occurred in apical shoot explants (Figure 3). This array of callogenic response of the explants for callus induction followed by its subsequent growth remained unaltered irrespective of plant growth regulators (PGRs) used in the medium. MS_0 and $0.5MS_0$ each supplemented with a combination of 0.50 ppm IAA and 0.50 ppm IBA, MS_0 and $0.5MS_0$ each supplemented with 0.50 ppm IAA and MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin proved to impart better callogenic effect in lavender than other combinations (Table 1, Figure 4). Comparatively, apical shoot explants exhibited best callogenic capacity compared with other explants. It also exhibited quickest callogenic response (callus induction followed by its growth) in MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin giving rise to friable yellowish callus. With all these notable advantages, callus cultures derived from apical shoot explants was unanimously carried forward for establishing cell suspension culture.

3.3 Cell suspension culture

The friable callus obtained from the apical shoot explants (Figure 5c) growing on the semisolid MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin were used to establish suspension cultures (Figure 5f). 1 g of this callus was transferred into 100 ml liquid MS (without agar and PGRs) medium and incubated on a rotary shaker with constant agitation (70 rpm) in the dark until individual cells of the callus could be observed as a uniform suspension in the liquid medium.

3.4 Suspension culture mediated callogenesis

A volume of 20 µl from this cell suspension culture (Figure 5f) was transferred onto the already screened, most effective, callus inducing semisolid medium i.e. MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin (Figure 5g). This was materialised to ensure that a uniform mass of callus be produced (Figure 5h) and utilized for every experiment(s) related to indirect regeneration and to have an accurate estimation of the frequency of regenerants to be produced from callus per media combination(s) as laid down in the subsequent experiments.

3.5 Caulogenesis

The suspended cells present in the liquid MS medium when transferred to MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin, led to prolific growth of a uniform callus mass weighing 20 g (approx). The callus obtained through direct mode (Figure 5e) as well as through cell suspension mediated mode (Figure 5h) were identical to each other in nature and appearance. However, the later was unambiguously more effective for an accurate numeric estimation of indirect regeneration response.

This callus mass weighing 20 g (approx) was divided into four equal parts weighing 5 g (approx) each, that were subcultured onto shoot inducing mediums with a specific distribution pattern (Distribution 1 - 5) as represented in figure(6).

The morphogenesis of adventitious buds from callus (derived through cell suspension mediated mode) were discernible within three weeks of culture when the callus was subjected to shoot inducing medium(s). MS_0 supplemented with BAP and Kin exhibited better caulogenic response than rest of the combinations of PGRs used alone or with $0.5MS_0$. The best combination of medium with prolific organogenic frequency (considering growth and development of adventitious shoots arising from callus) was that of MS_0 supplemented with 1.00 ppm BAP and 0.50 ppm Kin followed by MS_0 supplemented with 1.00 ppm BAP and 0.25 ppm Kin, MS_0 supplemented with 2.00 ppm BAP and 0.25 ppm Kin and MS_0 supplemented with 2.00 ppm BAP and 0.50 ppm Kin (Figure 7a-k). Least caulogenic response was exhibited by $0.5MS_0$ medium. A detailed description of the caulogenic response with respect to media combinations are represented in supplementary table (1).

3.6 Rhizogenesis

Indirect regeneration of lavender shoots from cell suspension culture mediated calli came into prominence within six weeks of subculturing. These regenerated shoots grew and developed nodes, leaves and in some cases primary branches. The healthy regenerants were excised and subcultured onto root inducing mediums. The $0.5MS_0$ supplemented with 0.50 ppm IBA exhibited best rhizogenic response in terms of quickest induction of roots as well as development of branches during root growth and development (Figure 8a-l). Hence, indirect regeneration of complete *in-vitro* plantlets of lavender was achieved.

At the same time, vegetative cuttings of the apical shoot meristems from the same donor lavender plant (used earlier for *in-vitro* establishment) growing in glasshouse condition were subjected to rooting in (1:2)sand-soil mixture. These lavender plants growing in glasshouse condition would subsequently act as checks during the eventual analysis of variation in the somaclones.

3.7 Acclimatization and hardening

The most healthy and complete *in-vitro* plantlets (indirectly regenerated somaclones) were selected to withstand and survive the inevitable stress of acclimatization and hardening processes. As a result, twenty nine acclimatized somaclones (Figure 9) along with two checks were gradually transplanted into small pots containing 2:1:1:1 mixture of soil: sand: vermiculite: farmyard manure and hardened in the glasshouse condition-1 (temperature range ≈20.00° C-23.00°C; humidity ≈36.80-40.00%). During the initial days of glasshouse transfer, these plantlets were irrigated with liquid $0.5MS_0$ without sucrose as and when needed. Later, tap water was used for irrigation. Twenty plantlets along with two checks were able to attain best vegetative growth and development (Figure 10) and were transferred to bigger pots containing 2:1 mixture of sand: soil and further hardened in the glasshouse condition-2 (temperature range ≈30.00°C-32.00°C; humidity ≈40.00-50.00%) with precautions for minimum root damage. Neither the somaclones nor the checks attained reproductive phase and never bloomed post glasshouse transfer.

3.8 Estimation of variation in somaclones

Quantitative data in the aspects of morphometric and chemometric traits of potted lavender plants (treatments) growing in glasshouse condition were recorded.

IV. MORPHOMETRIC DATA ANALYSIS

Data for six morphometric traits like plant height (T1), number of branches (T2), branch length (T3), fresh weight of plants (T4), root length (T5) and stem circumference (T6) were recorded and subjected to descriptive statistics (Table 2) ANOVA single factor (age of the plant) results indicated significant variation (MSS between groups=1911.26**) in the six morphometric traits (T1-T6) within the twenty two treatments (SC 1-20 and Control 1-2) at 95% level of confidence represented by table(3) and figure($10a_f$ - g_f). As ANOVA indicated statistical significance, post-hoc tests were used to distinguish significant differences between traits as follows:

4.1 Tukey-Kramer Post Hoc test

There were fifteen paired comparisons made for six traits [n(n-1)/2, where n=number of traits] All the paired comparisons were significantly different at α=0.05, except three paired combinations viz. number of branches-root length, number of branches-branch length and root length-branch length. But, at α=0.01 and 0.001, plant height-plant fresh weight paired trait became non-significant along with the aforesaid three traits.

4.2 Scheffe Post Hoc test

There were fifteen paired comparisons made for six traits [n(n-1)/2, where n=number of traits] All the paired comparisons were significant at α=0.05 and Scheffe's critical value=0.457. These paired comparisons remained significant even at α=0.01 and Scheffe's critical value=0.633 However, at α = 0.001 and Scheffe's critical value=0.880, the only paired trait viz. number of branches-branch length became non-significant.

4.3 Bonferroni corrected Student's T Post Hoc test

There were fifteen paired comparisons made for six traits [n(n-1)/2, where n=number of traits] All the paired comparisons were significant at α = 0.05, except three paired combinations viz number of branches-root length, number of branches-branch length and root length-branch length.

Therefore, Tukey-Kramer and Bonferroni corrected Student's T proved to be more conservative Post Hoc tests (exhibiting 80% significance each, at α=0.05) compared with Scheffe Post Hoc test (exhibiting 100% significance, at α=0.05) in relation of this portion of the study.

4.4 Correlation analysis

The correlation study (Figure 11, Module2: area FDEF**)** represented highest significant positive interaction between plant height-branch length(0.499**), followed by higher significant positive interactions between branch length-number of branches(0.456**), number of branches-plant height(0.427**), number of branches-plant fresh weight(0.412**), followed by high significant positive interactions between plant fresh weight-branch length(0.316**), number of branches-stem circumference (0.310**), plant height-stem circumference (0.305**), followed by significant positive interactions between number of branches-root length (0.223**), root length-branch length (0.128**), stem circumference-branch length (0.101**), stem circumference-plant fresh weight (0.076**), plant fresh weight-root length (0.049**).

The correlation study also represented highest significant negative interaction between root length-stem circumference(-0.529**), followed by plant height-root length(-0.180**) and plant height-plant fresh weight(-0.145**).

4.5 Regression analysis

The regression equation may be represented by all six traits (T1-T6) considered in this study, however the best fit model was represented by the number of branches having the best r^2 value(0.60), highest adjusted r^2 value (0.48) and best *p value* (0.007) as reflected in the figure (12). Regression equation based on the regression analysis (Figure 12) is $\hat{Y} = -26.76 + 0.76x_1 + 0.40x_2 + 0.37x_3 + 3.95x_4 - 0.02x_5$. This regression model suggests that for every unit change in plant height(x_1), root length(x_2), plant fresh weight(x_3), stem circumference(x_4) and branch length(x_5) the

number of branches(Ŷ) is going to be affected by 0.76 times, 0.40 times, 0.37 times, 3.95 times and 0.02 times respectively.

4.6 Chemometric Data Analysis

Morphometric data analysis was followed by essential oil estimation from lavender treatments (twenty somaclones and two checks). As aromatic flowering spikes were unavailable, leaves were sampled from all over the plant from every treatment and were subjected to HS-GC-MS analysis. Analysis of the chromatograms (Figure 13) confirmed that the best fifteen components of the essential oil (denoted by F1 to F15) which are under the present study represented 46.39% - 87.96% of the total essential oil components present in the treatments. This range, itself throws a lot of light to the presence of variation in the essential oil components in the treatments involved.

The somaclone SC5 exhibited the best concentration of three components of essential oil viz. D-Limonene (17.42%), α-Pinene (8.62%) and o-Cymene (5.69%) followed by SC6 exhibited the best concentration of two components of essential oil viz. Camphene (16.35%) and (+)-2-Bornanone (15.00%) and SC10 exhibited the best concentration of two components of essential oil viz. Cyclobutane, 1,2-dicyclopropyl- (8.61%) and α-Phellandrene (6.09%). SC1, SC3, SC7, SC8, SC12, SC18, Control1 and Control2 each exhibited the best concentration of single component of essential oil viz. 3-Carene (22.52%), β-Phellandrene (27.39%), Linalool (1.04%), Linalyl acetate (0.19%), p-Cymene (4.72%), Bicyclo [2.2.1] heptan- 2-ol,1,7,7- trimethyl-, (1S-endo)- (25.56%), Eucalyptol (59.45) and endo-Borneol (9.38%) respectively as reflected in the table (4).

Data for fifteen essential oil components represented by (F1 to F15) were recorded and subjected to descriptive statistics (Table 2). The ANOVA single factor (age of the plant) results indicated significant variation (MSS between groups=949.32**) in the fifteen traits (F1-F15) within the twenty two treatments (SC1-20 and Control1-2) at 95% level of confidence represented by table (5).

4.7 Correlation analysis

The correlation study (Figure 11, Module1: area ABFA) represented highest significant positive interaction between Camphene-(+) with 2-Bornanone(0.680**), followed by higher significant positive interactions between Eucalyptol with endo-Borneol (0.667**), Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl-, (1S-endo)- with Linalool(0.604**), α-Pinene with Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl-, (1S-endo)- (0.581**), β-Phellandrene with Linalyl acetate (0.533**), 3-Carene with α-Phellandrene (0.505**) followed by high significant positive interactions between Camphene with Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl-, (1S-endo)- (0.0.493**), Camphene with Linalool (0.490**), o-Cymene with Limonene(0.482**), followed by significant positive interactions between α-Pinene with D-Limonene (0.459**), 3-Carene with β-Phellandrene (0.402**).

The correlation study also represented highest significant negative interaction between p-Cymene with o-Cymene(-0.864**), followed by 3-Carene with (+)- 2-Bornanone(-0.725**) and endo-Borneol with Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl-,(1S-endo)-(-0.664**).

4.8 Regression analysis

The regression equation may be represented by all fifteen traits (F1-F15) considered in this study, however the best fit model was represented by (+)-2-Bornanone having the best R square value(0.981), highest adjusted R square value(0.944) and *p value*(0.72) as reflected in the table (6). Regression equation based on the regression analysis (Table 6) is $\hat{Y} = 8.556 + 0.29x_1 - 0.02x_2 - 9.13x_3 - 0.05x_4 - 0.26x_5 - 0.42x_6 + 0.32x_7 - 0.19x_8 + 0.17x_9 + 0.37x_{10} + 0.04x_{11} - 0.25x_{12} + 2.39x_{13} + 0.03x_{14}$. This regression model suggests that for every unit change in α-Phellandrene(x_1), β-Phellandrene (x_2), Linalyl acetate (x_3), Eucalyptol (x_4), endo-Borneol (x_5), 3-Carene (x_6), Camphene (x_7), α-Pinene (x_8), p-Cymene (x_9),

o-Cymene (x_{10}), Cyclobutane,1,2-dicyclopropyl- (x_{11}), Bicyclo [2.2.1] heptan- 2-ol,1,7,7- trimethyl-, (1S-endo)- (x_{12}), Linalool (x_{13}) and D-Limonene (x_{14}) the (+)-2-Bornanone ($\hat{Y}$) is going to be affected by 0.29 times, 0.02 times, 9.13 times, 0.05 times, 0.26 times, 0.42 times, 0.32 times, 0.19 times, 0.17 times, 0.37 times, 0.04 times, 0.25 times, 2.39 times and 0.03 times respectively.

4.9 Total data analysis of morphometric and chemometric traits

Convincing presence of variability in the morphometric traits (T1 to T6) and chemometric traits (F1 to F15) within the twenty two treatments (20 somaclones and 2 checks) were proved. This further opened the opportunity to statistically analyse the existence of variation within these 22 treatments with respect to twenty one cumulative morpho-chemo-metric traits (F1 to F15 and T1 to T6).

Data for twenty one traits were recorded and subjected to descriptive statistics (Table 2). The ANOVA single factor (age of the plant) results indicated significant variation (MSS between groups =1850.63**) in the twenty one traits (F1 to F15 and T1 to T6) for twenty two treatments (SC 1-20 and Control 1-2) at 95% level of confidence represented by table (7). As ANOVA indicated statistical significance, post-hoc tests were used to distinguish significant differences between traits as follows:

4.10 Tukey-Kramer Post Hoc test

There were 210 paired comparisons made for 21 traits [n(n-1)/2, where n= number of traits]. Among these, 124 paired comparisons were significantly different while the remaining 86 paired comparisons were not, at $\alpha = 0.05$. So, Tukey-Kramer Post Hoc test was successful in isolating 59.05% significantly different paired comparisons.

4.11 Scheffe Post Hoc test

There were 210 paired comparisons made for 21 traits [n(n-1)/2, where n= number of traits]. Among these, 207 paired comparisons were significantly different while the remaining 3 paired comparisons (endo-Borneol - Cyclobutane, 1,2-dicyclopropyl-; p-Cymene - (+)-2-Bornanone; o-Cymene - Cyclobutane,1,2-dicyclopropyl-) were not, at $\alpha = 0.05$ and Scheffe's critical value=0.079. So, Scheffe Post Hoc test was successful in isolating 98.57% significantly different paired comparisons.

4.11 Bonferroni corrected Student's T Post Hoc test

There were 210 paired comparisons made for 21 traits [n(n-1)/2, where n= number of traits]. Among these, 145 paired comparisons were significantly different while the remaining 65 paired comparisons were not at $\alpha = 0.05$. So, Bonferroni corrected Student's T Post Hoc test was successful in isolating 69.05% significantly different paired comparisons.

Therefore, Tukey-Kramer proved to be most conservative Post Hoc test than medium conservative Bonferroni corrected Student's T post hoc test and least conservative Scheffe Post Hoc test at $\alpha = 0.05$ in relation to cumulative assessment of twenty one morpho-chemo-metric traits of twenty two treatments in the present study. This idea is briefly represented with the help of Set Theory in table (8).

4.12 Correlation analysis

The correlation study (Figure 11) may be divided into three modules: Module 1- corresponds to chemometric correlation values demarcated by the large triangular area ABFA, Module 2- corresponds to morphometric correlation values demarcated by the small triangular area FDEF and Module 3- corresponds to morpho-chemo-metric correlation values demarcated by the rectangular area BCDFB. The results of correlation for module 1 and module 2 has already been discussed. However, module 3 is of particular interest as these values depict the combined interaction of morphometric and chemometric traits.

Module 3

The highest significant positive interaction is present between Linalyl acetate with stem circumference (0.503**), followed by high significant positive interactions between α-Phellandrene with plant height (0.380**), D-Limonene with root length (0.364**), o-Cymene with fresh weight of plants (0.353**), 3-Carene with stem circumference (0.325**), α-Phellandrene with branch length (0.308**), p-Cymene with branch length (0.302**), followed by significant positive interactions between o-Cymene with stem circumference (0.293**), α-Pinene with root length (0.283**), β-Phellandrene with number of branches (0.272**).

The correlation study also represented highest significant negative interaction between Bicyclo [2.2.1] heptan-2-ol,1,7,7-trimethyl-, (1S endo) with stem circumference (-0.519**), followed by p-Cymene with stem circumference (-0.444**) and 3-Carene with root length (-0.442**).

Overall correlation

The overall correlation taking module 1, 2 and 3 into account, reflects that the highest positive significant correlation existed between Camphene with (+)-2-Bornanone (0.680**) and the highest negative significant correlation existed between p-Cymene with o-Cymene (-0.864). The overall maxima and the minima both lie in the module 1, represented by chemometric correlation and demarcated by the area ABFA.

V. DISCUSSION

In the perspective of agro-climatic conditions of Jammu and Kashmir(INDIA), lavender has been predominantly grown as a vegetatively propagable crop since it was introduced in INDIA. The unavoidable clonal propagation practice with lavender germplasm on one hand gave rise to negligible or no genetic variation at all and on the other hand amplified the chance of genetic erosion. The scarcity of genetic variation in the existing germplasm was possibly one of the major bottlenecks for the failure of varietal improvement programs mediated through conventional approaches in regard to lavender genetic improvement in Indian context. A closer introspection to the problem led to a convincing solution - inducing significant variation *in-vitro* using PTC methods(indirect regeneration) followed by selection of putative candidates. This idea was seldom conceived by former and present experts, but was brought to reality with proper investigations in the present study.

As a regular observation, lavender plants donot attain reproductive phase in the experimental location, hence, seeds as explants were unavailable to initiate the process of *in-vitro* establishment. Alternatively, apical shoot meristems were used to establish a stock of sterile cultures in MS_0 medium (Al Khateeb et al. 2017).

There has been a number of studies in the aspect of micropropagation of *Lavandula sp* in the recent past. In the present study, sterile *in-vitro* cultures of lavender have been quickly established by utilizing those findings for the purpose of inducing genetic variation in the resulting complete plantlets (somaclonal variants). The PGRs - BAP and Kin are proclaimed as multiple shoot producers (Yew et al. 2010). Hence, different combinations of them were used in MS_0 and $0.5MS_0$ medium for multiple shoot proliferation in lavender. The best medium for the rapid proliferation of microshoots was MS_0 supplemented with 1 ppm BAP and 0.50 ppm Kin. Unlike the present results for *L. angustifolia*, Al-Bakhit et al. (2007) estimated a much lower concentration of BAP to be most effective for the proliferation of *L. latifolia* microshoots. In another experiment, Zuzarte et al. (2010) confirmed that adding BAP induced higher number of microshoots in *L. pendunculata*. Synergistic effect of higher concentration of BAP and TDZ was most effective for propagation of microshoots in *L. vera* was advocated by Andrade et al. (1999).

The apical shoot explants growing in MS_0 supplemented with 5 ppm 2,4-D and 0.25 ppm Kin exhibited quickest response to callus induction followed by vigorous proliferation of friable yellowish calli. Unlike the present findings, Keykha et al. (2014) used leaf explants to generate callus and observed 2 ppm 2,4-D and 2 ppm BAP

in dark conditions to be the most suitable condition for the callus induction and its growth. Falk et al. (2013) advocated that MS medium supplemented with TDZ to be the best combination for callus induction in *L. angustifolia*.

A cell suspension culture of lavender was established using a known mass of the yellowish friable callus derived from apical shoot meristems. The callus remained in liquid MS_0 (without agar and PGRs) being continuously agitated by circular motion until a uniform suspension of cells was formed. Lappin et al. (1987) also derived the same results like the present study while establishing cell suspensions of *L. angustifolia* from callus induced on agar-solidified MS medium supplemented with 2,4-D and Kin. Watanabe et al. (1982) also established suspension cultures of green *L. vera* cells to screen high vitamin producing cells.

Callus cultures were again generated using a known volume of the suspension culture. The calli so obtained from suspension cultured mediated mode was similar in all respects to the calli derived from apical shoot explants mode. However, the only difference was that, the former was quantifiable but the later wasn't.

The quantifiable mass of calli was distributed in five patterns onto each shoot inducing mediums for indirect regeneration of shootlets. The purpose of this distribution was to ensure maximum exposure of callus cells to the medium. MS_0 supplemented with 1.00 ppm BAP with 0.50 ppm Kin proved to be the best combination for caulogenesis. Synergistic effect of BAP and Kin was evident in the present study unlike Adesoye et al. (2012) in *Sphenostylis stenocarpa*, Al Khateeb et al. (2013) in *Moringa peregrina*, Ahmad and Anis (2014) in *Vitex trifolia*. Formation of complete plantlets occurred after rhizogenesis in $0.5MS_0$ supplemented with 0.50 ppm IBA. The most healthy and complete plantlets (somaclones) were put under the inevitable stress of acclimatization and hardening. The resultant somaclones were analysed for morphometric and chemometric variations using statistical tools and techniques.

Six measurable morphological features (morphometric traits) were selected. Statistical analysis of morphometric data was focused mainly on detection of variation(s) induced within the somaclones as a result of indirect regeneration approach. One way ANOVA exhibited significant differences among the morphometric traits with 99.9% confidence. Three follow up post-hoc test (viz. Tukey-Kramer, Scheffe and Bonferroni corrected Student's T) were also performed to detect pair-wise significant differences at 95% confidence level. The purpose of three post-hoc tests was fulfilled when the most effective post-hoc test could be identified among them. Tukey-Kramer and Bonferroni corrected Student's T both proved to be equally most effective post-hoc tests. Tukey-Kramer multiple comparison test was used to distinguish significantly different treatments following the ANOVA test in the studies on *Brassica napus* by Akasaka-Kennedy et al. (2005), on *Grateloupia dichotoma* by Yokoya and Handro (1996) and on *Scilla natalensis* by McCartan, and Van Staden (1998). Tukey-Kramer and Dunnet tests were performed on *Cryptanthus sinuosus* by Arrabal et al. (2002) and on *Caladium bicolor* by Ahmed et al. (2002).

In the absence of flowering spikes, fifteen detectable constituents of essential oil (chemometric traits) from leaves were sampled from each treatment. Similar studies of using leaves in place of or alongwith flowing spikes have been reported by Hassanpouraghdam et al. (2011) in *L. officinalis*, Aburjai et al. (2005) in *L. coronopofolia* and Cristina et al. (1995) in *L. pinnata*. Statistical analysis of chemometric data was focused mainly on detection of variation(s) in the essential oil profile induced within the somaclones as a result of indirect regeneration approach. One way ANOVA exhibited significant differences among the chemometric traits with 99.9% confidence. Correlation and multiple regression analysis have also contributed in explaining the effects of one trait on another. A combined statistical analysis of variance (one way ANOVA) encompassing sumtotal traits exhibited significant difference with 99.9% confidence. Three follow up post-hoc test (viz. Tukey-Kramer,

Scheffe and Bonferroni corrected Student's T) were also performed to detect pair-wise significant differences at 95% confidence level. The purpose of three post-hoc tests was fulfilled when the most effective/suitable post-hoc test could be identified among them. Tukey-Kramer proved to be most conservative post-hoc test when compared with Bonferroni corrected Student's T post-hoc test and Scheffe post-hoc test in relation to assessment of 21 morpho-chemo-metric traits of 22 treatments in the present study. Application of Bonferroni's correction on Student's T test improved the conservativeness of mere Student's T test result from 76.67% to 69.05%. Even then Tukey-Kramer's post-hoc values (59.05%) were far more encouraging than the rest. Tukey-Kramer multiple comparison test was used to distinguish significantly different treatments following the ANOVA test in the studies on *Portulaca grandiflora* by Khandare et al. (2011), on *Lavandula dentata* by Echeverrigaray et al. (2005), on *Allium sativum* by Robledo-Paz et al. (2000) and on *Thapsia garganica* by Makunga et al. (2003).

VI. CONCLUSIONS

Quantifiable callus cultures raised through cell suspension culture mediated route was instrumental in the indirect regeneration of lavender. Complete somaclonal plantlets were raised, selected and subjected to glasshouse establishment where variation in twenty one morphometric and chemometric traits were analysed for twenty somaclones and two checks. Significant amount of variation existed in all the somaclones raised through *in-vitro* approach which was precisely proved by more than one statistical tests. SC5 (Figure 14) proved to be the most putative somaclonal variant followed by SC6 and SC10.

6.1 Upcoming experiments and hypothesis

The putative somaclonal variants (SC5, SC6 and SC10) are now under vegetative luxuriance along with the rest of the somaclonal variants. These variants will be clonally propagated in the glasshouse to build a population of plants and will be transferred to that habitat which may eventually lead to the reproductive expression. Selection of early maturing lines, with higher inflorescence count may be obtained from field study which will be carried out soon.

ACKNOWLEDGEMENT

The authors are thankful to Late Dr. Suresh Chandra, CSIR-IIIM, Jammu for his continuous encouragement. Thanks are also due to Dr. Rekha Sapru Dhar, Principal Technical Officer, Plant Tissue Culture Department and Mr. Amit Kumar, Technical Assistant, Instrumentation Division, CSIR-IIIM, Jammu for providing enthusiasm and support during the research. The authors also thank the Director, CSIR-IIIM, Jammu, India, for providing the necessary facilities during the course of this investigation.

Conflict statement

No conflict of interest is declared by the authors

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Table 1: Media combinations and their effect on growth and development in *Lavandula angustifolia* Mill

Sl No	Media composition	*In-vitro* establishment and multiplication of cultures.	Callogenesis	Caulogenesis	Rhizogenesis
1	$0.5MS_0$	Least growth response	-NA-	Least response	Better response
2	$0.5MS_0$+0.50 ppm IBA	-NA-	No response	-NA-	Best response
3	$0.5MS_0$+0.50 ppm IAA	-NA-	Better response	-NA-	-NA-
4	$0.5MS_0$+0.50 ppm IAA+0.50 ppm IBA	-NA-	Better response	-NA-	-NA-
5	MS_0	75% of the explants completely recovered from initial inoculation stress within 2 weeks.	-NA-	Low response	Good response
6	MS_0+1 ppm BAP	Moderate growth response	-NA-	Moderate response	-NA-
7	MS_0+2 ppm BAP	Moderate growth response	-NA-	Good response	-NA-
8	MS_0+ 0.25 ppm Kin	Moderate growth response	-NA-	Low response	-NA-
9	MS_0+ 0.50 ppm Kin	Good growth response	-NA-	Moderate response	-NA-
10	MS_0+1 ppm BAP+ 0.25 ppm Kin	Better growth response	-NA-	Better response	-NA-
11	MS_0+1 ppm BAP+ 0.50 ppm Kin	Best overall growth response for fastest multiplication in minimum time.	-NA-	Best response	-NA-
12	MS_0+2 ppm BAP+ 0.25 ppm Kin	Better growth response	-NA-	Better response	-NA-
13	MS_0+2 ppm BAP+ 0.50 ppm Kin	Good growth response	-NA-	Good response	-NA-
14	MS_0+5 ppm 2,4-D+ 0.25 ppm Kin	-NA-	Best response in minimum time with apical shoot explants.	-NA-	-NA-
15	MS_0+0.50 ppm IAA	-NA-	Better response	-NA-	-NA-
16	MS_0+0.50 ppm IBA	-NA-	No response	-NA-	Good response
17	MS_0+0.50 ppm IAA+0.50 ppm IBA	-NA-	Better response	-NA-	-NA-

Where, -NA- = Not Applicable

Table 2: Descriptive features of morpho-chemo-metric traits of *L. angustifolia* Mill

	Morphometric traits						Chemometric traits													
Features	T1	T2	T3	T4	T5	T6	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
Mean ± Standard Error	24.86 ± 0.60	16.59 ± 0.98	14.59 ± 1.19	29.08 ± 1.15	2.11 ± 0.09	17.73 ± 0.88	23.59 ± 3.39	1.03 ± 0.55	11.64 ± 1.14	8.29 ± 0.82	4.62 ± 0.37	2.68 ± 0.33	1.30 ± 0.35	1.23 ± 0.59	13.90 ± 1.85	0.39 ± 0.06	3.05 ± 1.09	2.67 ± 0.65	0.77 ± 0.29	3.39 ± 1.87	0.01 ± 0.01
Standard Deviation	2.83	4.59	5.59	5.39	0.46	4.12	15.93	2.59	5.34	3.87	1.74	1.57	1.64	2.74	8.66	0.26	5.15	3.03	1.39	8.77	0.04
Sample Variance	8.03	21.11	31.25	29.15	0.21	16.99	253.73	6.74	28.36	14.97	3.04	2.47	2.68	7.53	75.07	0.07	26.51	9.16	1.93	76.87	0.002
Count	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22

Chemometric traits are : F1=Eucalyptol(%), F2= endo-Borneol(%), F3= 3-Carene(%), F4= Camphene(%), F5= α-Pinene(%), F6= p-Cymene(%), F7= o-Cymene(%), F8= Cyclobutane,1,2-dicyclopropyl-(%), F9= Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-(%), F10= Linalool(%), F11= D-Limonene(%), F12= (+)-2-Bornanone(%), F13= α-Phellandrene(%), F14= β-Phellandrene(%), F15= Linalyl acetate(%).

Table 3: ANOVA for six morphometric traits of *L. angustifolia* Mill. somaclones indicating significant variation between groups

Source of variation	Sum of Squares	Degrees of freedom	Mean Sum of Squares	F- value
Between groups	9556.33	5.00	1911.26***	107.43
Within groups	2241.59	126.00	17.79	
Total	11797.92	131.00		

*Where, *** = $p < 0.001$.*

Table 4: Essential oil constituents in somaclones of *L. angustifolia* Mill

Treatments	Concentrations (%) of essential oil constituents from leaves of lavender treatments.															Total %	Number of highest Constituent(s)/Treatment
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15		
SC1	0.00	0.00	22.52	5.74	3.26	3.10	0.00	0.00	9.48	0.50	0.00	0.48	2.83	0.00	0.00	47.91	1
SC2	20.50	0.00	12.30	11.32	6.06	4.15	0.00	0.00	19.89	0.39	0.00	1.68	0.78	0.00	0.00	77.07	0
SC3	13.15	0.00	16.01	6.61	3.39	3.65	0.00	0.00	10.30	0.00	0.00	0.55	1.32	27.39	0.00	82.37	1
SC4	0.00	0.00	14.41	6.09	3.25	3.11	0.00	0.00	17.28	0.29	0.00	0.83	1.13	0.00	0.00	46.39	0
SC5	11.35	0.00	15.05	0.00	8.62	0.00	5.69	0.00	8.92	0.20	17.42	1.43	0.68	0.00	0.00	69.36	3
SC6	22.47	1.49	0.00	16.35	0.00	0.00	2.78	0.00	0.00	0.44	5.05	15.00	0.61	0.00	0.00	64.19	2
SC7	10.54	0.00	18.15	9.65	5.22	3.84	2.29	0.00	20.86	1.04	0.00	1.93	1.01	0.00	0.00	74.53	1
SC8	22.70	4.11	16.66	3.12	2.98	1.24	1.99	0.00	0.00	0.41	0.00	0.00	1.24	24.32	0.19	78.96	1
SC9	8.82	0.00	18.28	8.47	5.59	2.40	1.39	0.00	12.85	0.43	0.00	0.90	1.19	22.93	0.00	83.25	0
SC10	15.04	0.00	15.29	7.93	4.63	3.57	0.00	8.61	19.51	0.47	0.00	1.93	6.09	0.00	0.00	83.07	2
SC11	21.31	0.00	8.28	10.85	5.02	1.34	2.78	0.00	25.29	0.86	7.57	3.39	0.00	0.00	0.00	86.69	0
SC12	39.91	0.00	15.00	9.39	5.39	4.72	0.00	0.00	8.14	0.19	0.00	1.87	0.00	0.00	0.00	84.61	1
SC13	14.91	0.00	8.91	11.27	5.63	1.35	2.88	0.00	21.15	0.48	13.41	3.42	0.00	0.00	0.00	83.41	0
SC14	29.69	0.00	3.84	11.01	5.45	4.37	0.00	0.00	22.92	0.38	5.68	4.62	0.00	0.00	0.00	87.96	0
SC15	18.46	0.00	7.24	12.34	6.23	4.51	0.00	0.00	23.87	0.71	9.73	3.92	0.00	0.00	0.00	87.01	0
SC16	41.51	0.00	8.48	6.26	5.08	4.34	0.00	0.00	11.58	0.22	0.00	1.74	0.00	0.00	0.00	79.21	0
SC17	20.83	0.00	10.60	13.19	5.93	1.48	3.07	7.45	17.95	0.38	0.00	3.76	0.00	0.00	0.00	84.64	0
SC18	21.62	0.00	7.26	10.88	5.03	4.05	0.00	0.00	25.56	0.55	8.28	3.61	0.00	0.00	0.00	86.84	1
SC19	41.53	0.00	12.44	4.81	3.99	3.00	0.00	4.57	9.66	0.27	0.00	1.02	0.00	0.00	0.00	81.29	0
SC20	28.32	0.00	10.27	9.52	5.43	0.00	3.21	6.39	20.68	0.26	0.00	2.58	0.00	0.00	0.00	86.66	0
Control 1	59.45	7.59	7.28	3.98	2.57	1.31	2.59	0.00	0.00	0.00	0.00	2.42	0.00	0.00	0.00	87.19	1
Control2	56.91	9.38	7.67	3.70	2.98	3.40	0.00	0.00	0.00	0.00	0.00	1.64	0.00	0.00	0.00	85.68	1

Where, SC= Somaclones, F1=Eucalyptol, F2= endo-Borneol, F3= 3-Carene, F4= Camphene, F5= alpha.-Pinene, F6= p-Cymene, F7= o-Cymene, F8= Cyclobutane, 1,2-dicyclopropyl-, F9= Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-, F10= Linalool, F11= D-Limonene, F12= (+)-2-Bornanone, F13= .alpha.-Phellandrene, F14= beta.-Phellandrene, F15= Linalyl acetate. Underlined numbers indicate highest values while underlined & italicized number indicate lowest values in the range.

Table 5: ANOVA for fifteen chemometric traits of *L. angustifolia* Mill. somaclones indicating significant variation between groups

Source of Variation	Sum of Squares	Degrees of freedom	Mean Sum of Squares	F- value
Between Groups	13290.41	14	949.315***	27.969
Within Groups	10691.50	315	33.941	
Total	23981.92	329		

*Where, *** = $p < 0.001$.*

Table 6: Regression table for best fit model related to chemometric traits of *L. angustifolia* Mill.

	Chemometric traits														
Regression Statistics	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
Multiple r	0.953	0.917	0.987	0.962	0.945	0.965	0.969	0.872	0.985	0.927	0.902	0.991	0.877	0.766	0.823
r^2	0.909	0.841	0.974	0.926	0.892	0.932	0.938	0.761	0.971	0.859	0.814	0.981	0.769	0.587	0.678
Adjusted r^2	0.725	0.523	0.921	0.778	0.676	0.796	0.815	0.282	0.913	0.578	0.441	0.944	0.309	-0.239	0.033
Standard Error	8.349	1.792	1.495	1.823	0.991	0.709	0.704	2.326	2.552	0.168	3.850	0.719	1.155	9.758	0.039
Observations	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
Model Rank(s)	-	-	2nd	-	-	-	-	-	3rd	-	-	1st	-	-	-

Where, Chemometric traits are : F1=Eucalyptol, F2= endo-Borneol, F3= 3-Carene, F4= Camphene, F5= alpha.-Pinene, F6= p-Cymene, F7= o-Cymene, F8= Cyclobutane, 1,2-dicyclopropyl-, F9= Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-, F10= Linalool, F11= D-Limonene, F12= (+)-2-Bornanone, F13= .alpha.-Phellandrene, F14= beta.-Phellandrene, F15= Linalyl acetate.

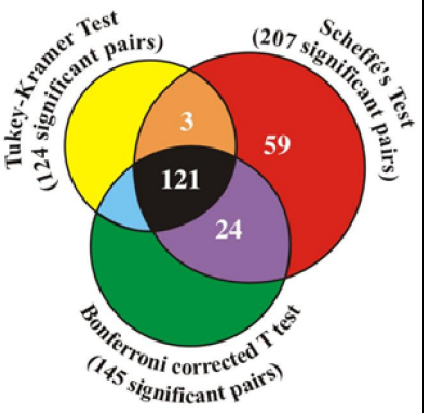
Table 7: ANOVA for twentyone (T1-T6 and F1-F15) morpho-chemo-metric traits of *L. angustifolia* Mill. somaclones indicating significant variation between groups.

Source of Variation	Sum of Squares	Degrees of freedom	Mean Sum of Squares	F- value
Between Groups	37012.519	20	1850.626***	63.104
Within Groups	12933.097	441	29.327	
Total	49945.616	461		

*Where, *** = $p < 0.001$.*

Table 8: Comparison of efficacy among three post hoc tests simplified through Set Theory.

Sl No.	Set :	Set notation :	Number of significant pairs(n) :	Information:	
1.	Tukey-Kramer post-hoc test	T	n(T)=124	59.05% significant pairs.	
2.	Scheffe's post-hoc test	S	n(S)=207	98.57% significant pairs.	
3.	Bonferroni corrected post-hoc T test	B	n(B)=145	69.05% significant pairs.	
4.	Common significant pairs between T, S and B	T∩S∩B, black zone	n(T∩S∩B)=121	-	
5.	Common significant pairs between T and S	T∩S, orange and black zone	n(T∩S)=124	-	
6.	Common significant pairs between S and B	S∩B, violet and black zone	n(S∩B)=145	-	
7.	Common significant pairs between T and B	T∩B, blue and black zone	n(T∩B)=121	(T∩B)= (T∩S∩B)=121	
8.	Common significant pairs only between T and S	Orange zone	n(T∩S) - n(T∩S∩B)= 124-121=3	-	
9.	Common significant pairs only between S and B	Violet zone	n(S∩B) - n(T∩S∩B)=145-121=24	-	
10.	Common significant pairs only between T and B	Blue zone	n(T∩B) - n(T∩S∩B)=121-121=0	ϕ	
11.	Number of elements of only T	Yellow zone	n(T) - {n(T∩B) ∪ n(S∩T)}=0	ϕ	
12.	Number of elements of only S	Red zone	n(S) - {n(T∩S) ∪ n(S∩B)}=59	-	
13.	Number of elements of only B	Green zone	n(B) - {n(T∩B) ∪ n(S∩B)}=0	ϕ	



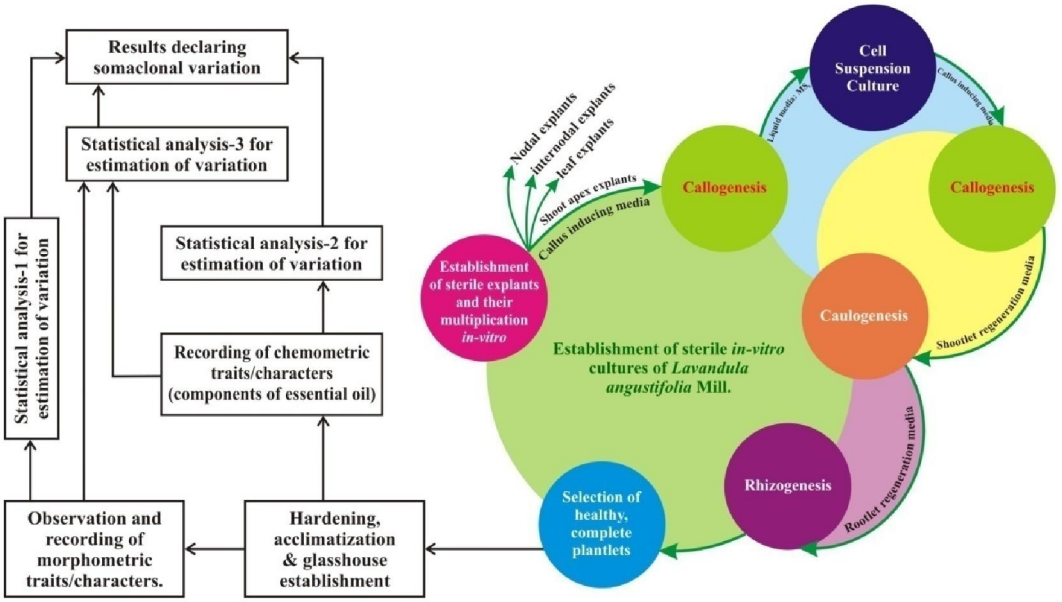


Figure 1: A schematic representation of the present study.

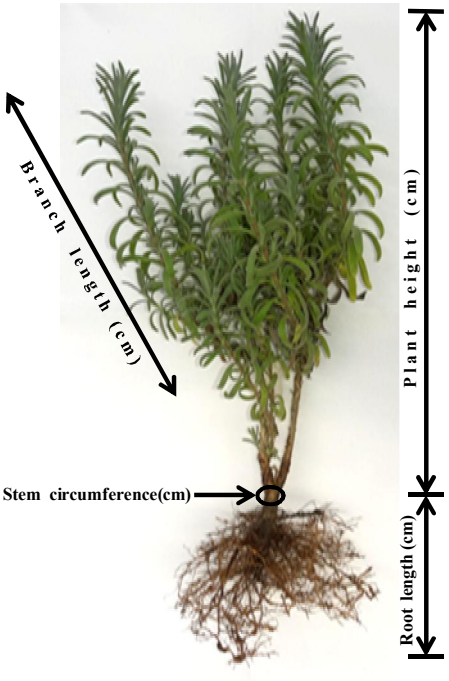
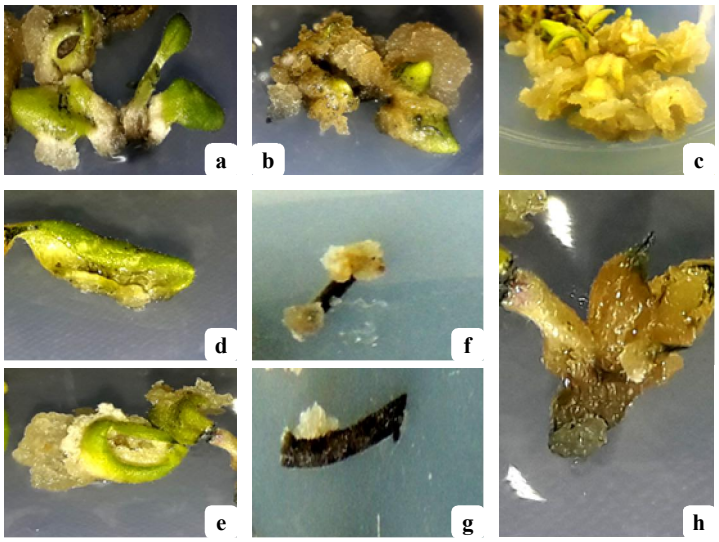
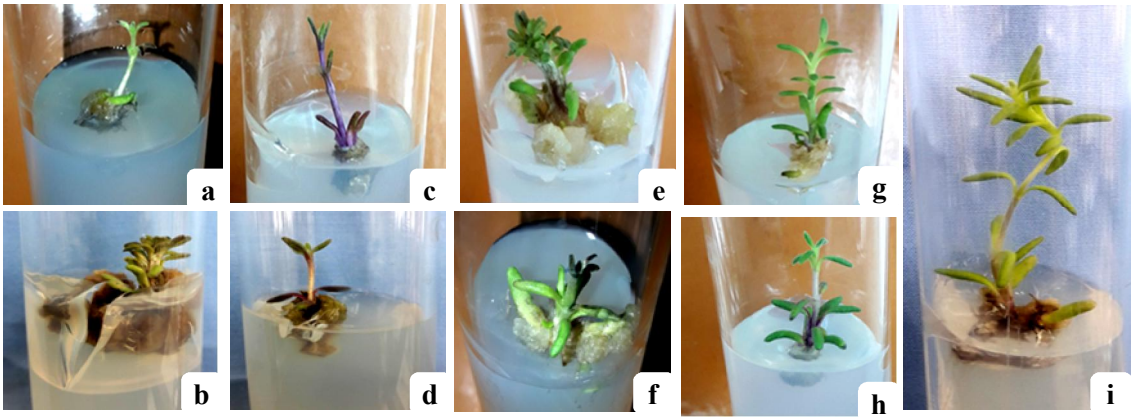


Figure 2: Some of the morphometric traits of *L. angustifolia* Mill. considered in our study.



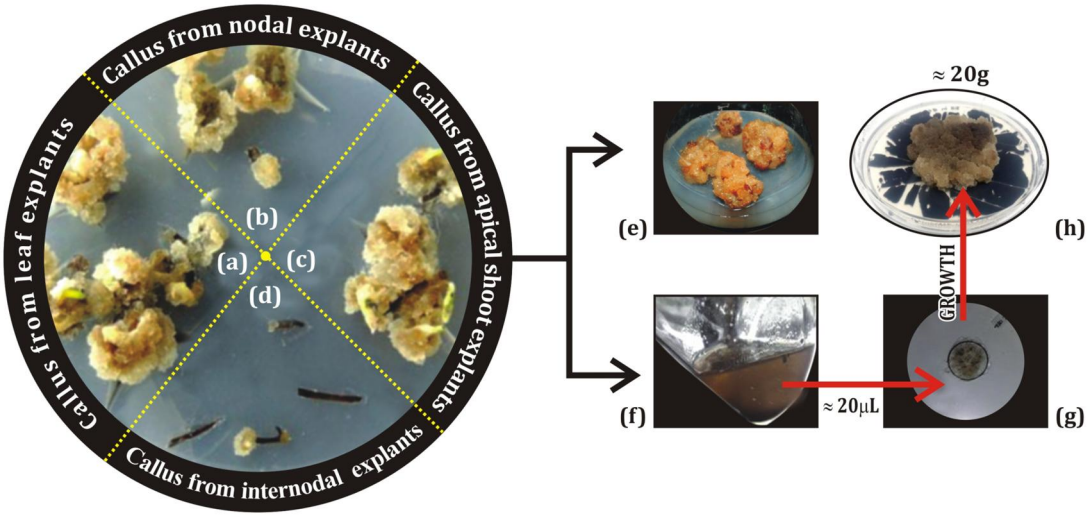
(a)-(c) apical shoot explants giving rise to vigorous callus, (d)-(e) leaf explants giving rise to callus, (f)-(g) internodal explants giving rise to inconspicuous callus and (h) nodal explant giving rise to callus.

Figure 3: Different stages of callogenesis of *L. angustifolia* explants.



(a)-(b) 0.5MS$_0$+0.5 ppm IAA+0.5 ppm IBA media composition, (c)-(d) 0.5MS$_0$+0.5 ppm IBA media composition, (e)&(g) MS$_0$+0.5 ppm IAA media composition, (f) 0.5MS$_0$+0.5 ppm IAA media composition, (h)-(i) MS$_0$+0.5 ppm IAA+0.5 ppm IBA media composition gave rise to callus.

Figure 4: Callogenic response in some media combinations on apical shoot explants of *L. angustifolia*.



Callus formation in progress from (a) leaf explants, (b) nodal explants, (c) apical shoot explants, (d) internodal explants, (e) Callus formation complete from apical shoot explants, which is used for other experiments not included in this study, (f) Callus from apical shoot explants used to develop cell suspension culture in liquid MS$_0$ medium (no agar and no PGRs), (g) 20µl of cell suspension culture transferred and evenly distributed on MS$_0$ + 5.00 ppm 2,4-D + 0.25 ppm Kin media composition, (h) a mass of callus (20 g) obtained from cell suspension culture.

Figure 5: Callogenic response of different explants in MS$_0$ + 5.00 ppm 2,4-D + 0.25 ppm Kin media composition of *L. angustifolia*.

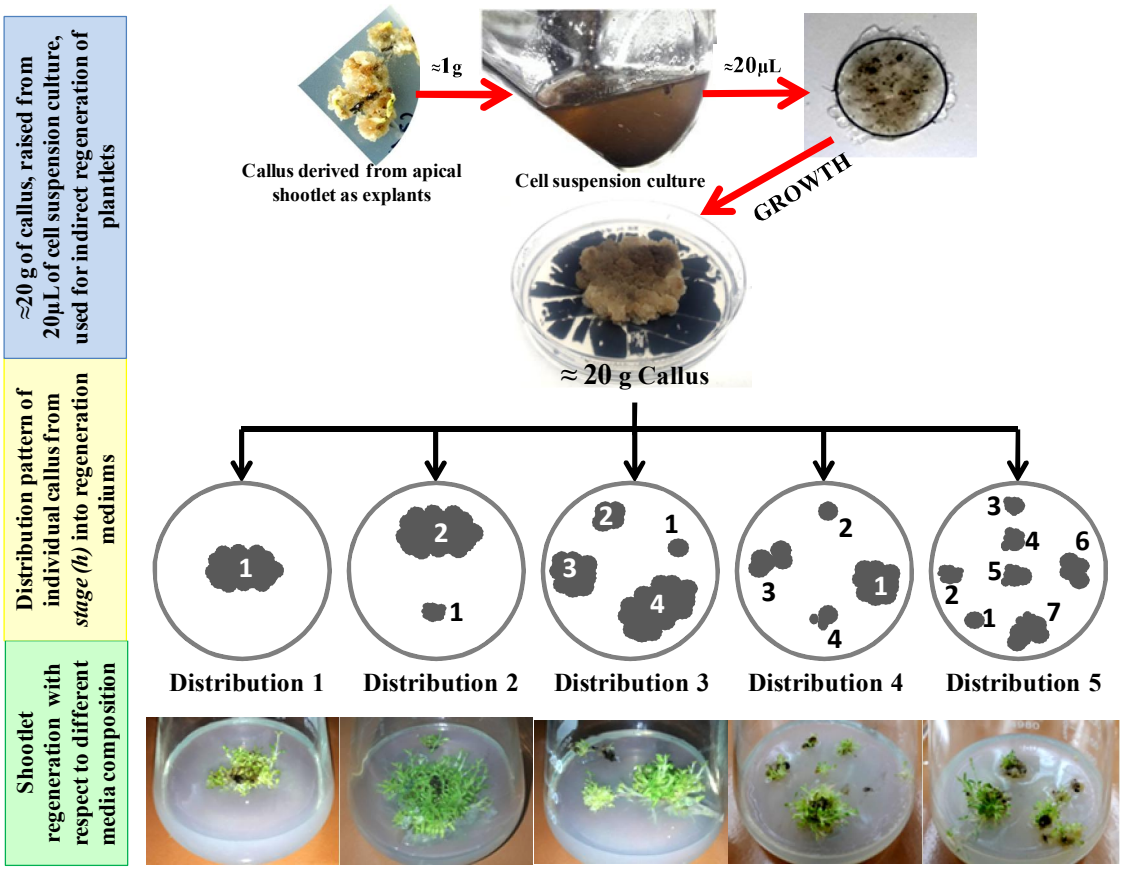
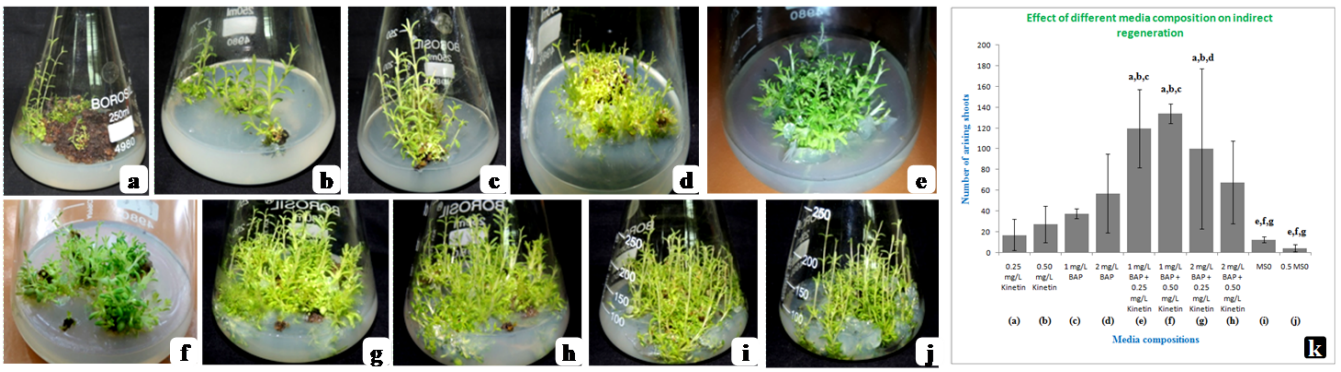
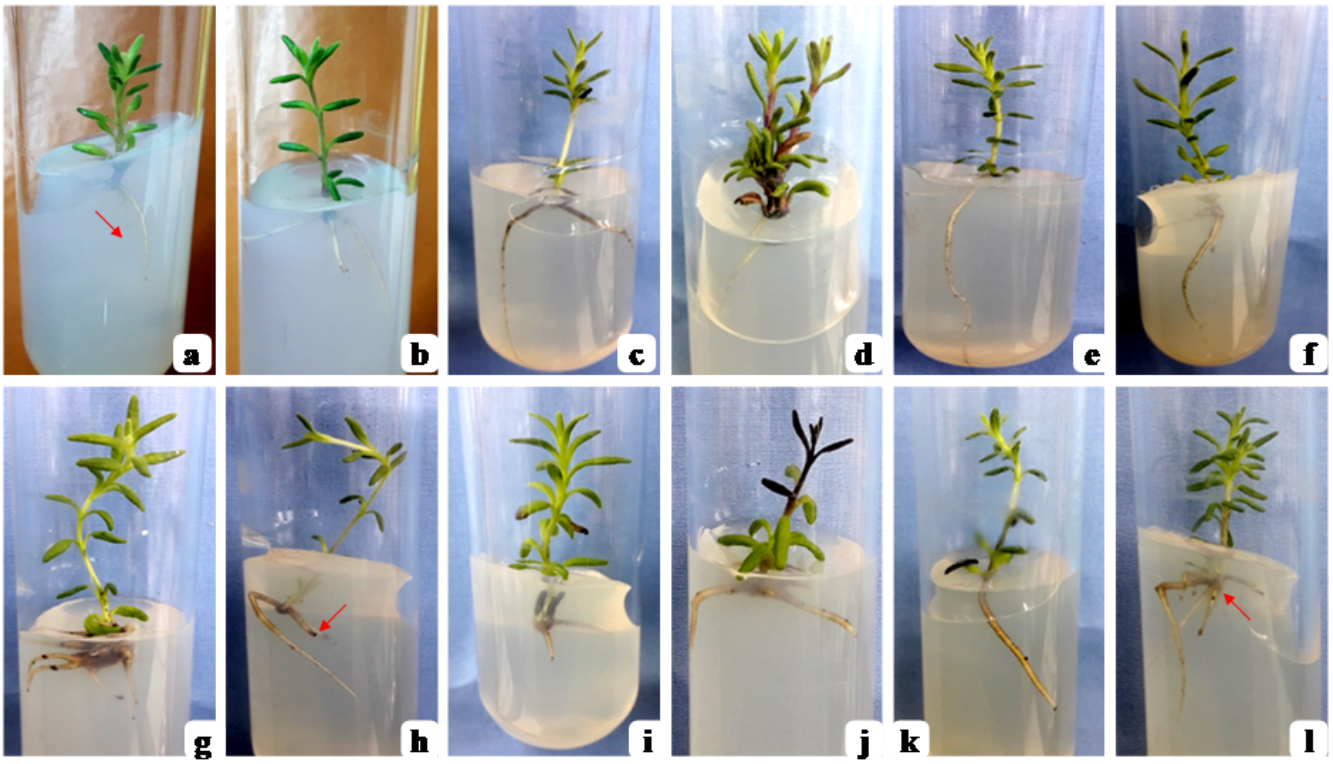


Figure 6: Caulogenic response in different distribution pattern in shoot inducing media composition of *L. angustifolia*.



(a) 0.50 MS_0, (b) MS_0, (c) MS_0 + 0.25 ppm Kin, (d) MS_0 + 0.50 ppm Kin, (e) MS_0 + 1.00 ppm BAP, (f) MS_0 + 2.00 ppm BAP, (g) MS_0 + 1.00 ppm BAP + 0.25 ppm Kin, (h) MS_0 + 1.00 ppm BAP + 0.50 ppm Kin, (i) MS_0 + 2.00 ppm BAP + 0.25 ppm Kin, (j) MS_0 + 2.00 ppm BAP + 0.50 ppm Kin, (k) an estimation of the number of shoots arising from different shoot regeneration media. The mean number of shoots from five replicates per medium combination are represented by respective columns and the error bars depict respective SD. The different letters above error bars indicate a statistically significant difference as per Tukey-Kramer procedure with significance level at $p<0.05$.

Figure 7: Caulogenic response in different media composition of *L. angustifolia*.



(a) 0.50 MS_0 + 0.50 ppm IBA, (b) 0.5 MS_0, (c) 0.5 MS_0, (d) MS_0, (e) 0.5 MS_0, (f) MS_0, (g) MS_0 + 0.50 ppm IBA, (h) 0.50 MS_0 + 0.50 ppm IBA, (i) MS_0 + 0.50 ppm IBA, (j) 0.5 MS_0, (k) 0.5 MS_0, (l) 0.50 MS_0 + 0.50 ppm IBA.

Figure 8: Rhizogenesis in different media composition of *L. angustifolia* leading to the formation of complete plantlets. Red arrows indicates development of primary branches in roots.

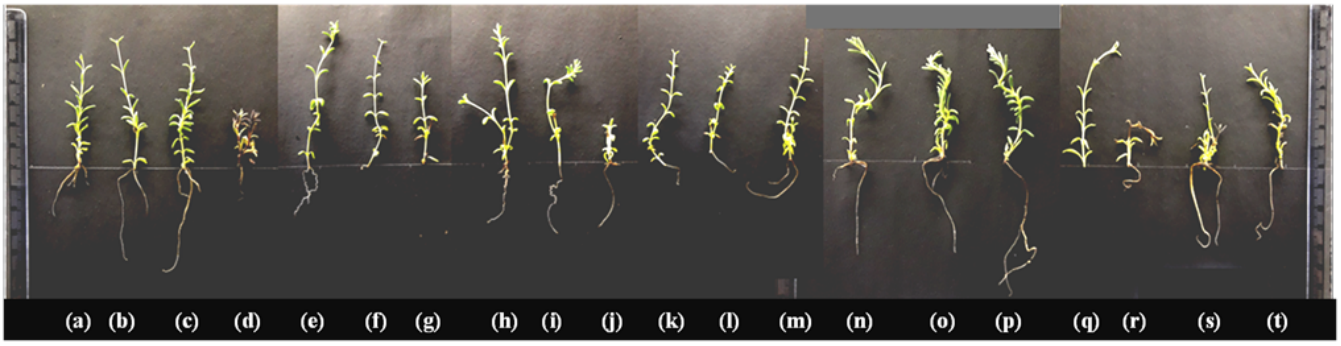
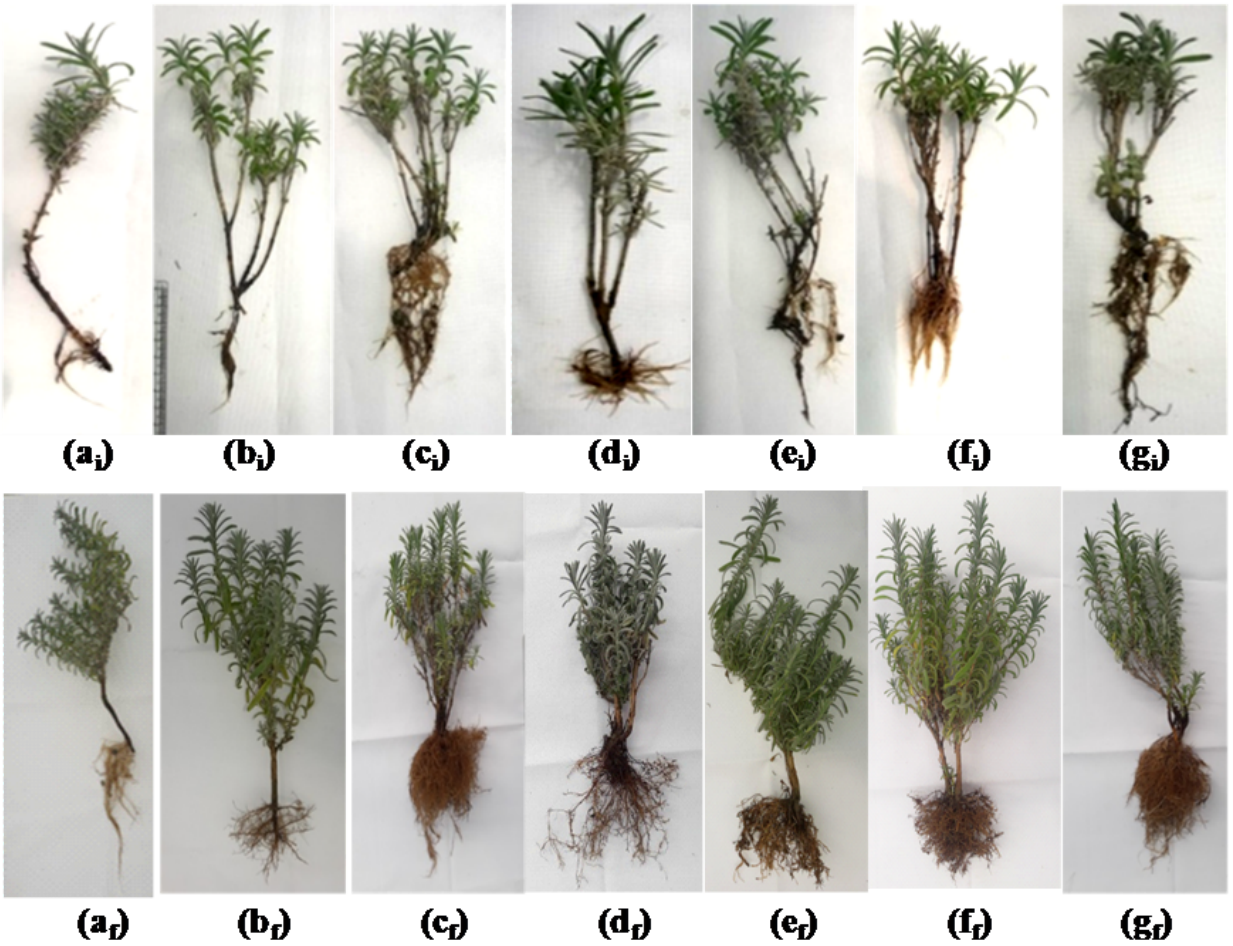


Figure 9: Some of the complete plantlets of *L. angustifolia* (somaclones) ready to go through acclimatization and hardening stress.



(a_i - g_i) somaclones during intermediate stage of growth and development, (a_f - g_f) same somaclones during final stage of growth and development.

Figure 10: Some representative somaclones of *L. angustifolia* under acclimatization and hardening stress.

		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	T1	T2	T3	T4	T5	T6
F1	A	1.000																				
F2		0.667 **	1.000																			
F3		-0.495 **	-0.230 **	1.000																		
F4		-0.183 **	-0.403 **	-0.481 **	1.000																	
F5		-0.213 **	-0.470 **	0.209 **	-0.058 **	1.000																
F6		0.059 **	-0.158 **	0.145 **	0.074 **	0.127 **	1.000															
F7		-0.104 **	0.029 **	-0.080 **	-0.062 **	0.243 **	-0.864 **	1.000														
F8		0.020 **	-0.185 **	0.061 **	0.113 **	0.120 **	-0.187 **	0.087 **	1.000													
F9		-0.432 **	-0.664 **	-0.055 **	0.493 **	0.581 **	0.289 **	-0.099 **	0.205 **	1.000												
F10		-0.504 **	-0.450 **	0.077 **	0.490 **	0.200 **	0.083 **	0.100 **	-0.042 **	0.604 **	1.000											
F11		-0.223 **	-0.219 **	-0.320 **	0.076 **	0.459 **	-0.291 **	0.482 **	-0.278 **	0.289 **	0.230 **	1.000										
F12		0.055 **	-0.025 **	-0.725 **	0.680 **	-0.401 **	-0.324 **	0.234 **	-0.036 **	-0.073 **	0.177 **	0.267 **	1.000									
F13		-0.436 **	-0.149 **	0.505 **	-0.154 **	-0.123 **	0.112 **	-0.209 **	0.396 **	0.008 **	0.117 **	-0.243 **	-0.179 **	1.000								
F14		-0.220 **	0.049 **	0.402 **	-0.236 **	-0.161 **	-0.051 **	-0.056 **	-0.181 **	-0.289 **	-0.187 **	-0.240 **	-0.293 **	0.142 **	1.000							
F15	B	-0.013 **	0.265 **	0.211 **	-0.299 **	-0.211 **	-0.205 **	0.094 **	-0.100 **	-0.358 **	0.022 **	-0.132 **	-0.197 **	0.076 **	0.533 **	1.000	F					
T1		0.022 **	0.212 **	0.158 **	-0.390 **	-0.253 **	0.219 **	-0.378 **	0.025 **	-0.291 **	-0.204 **	-0.182 **	-0.092 **	0.380 **	-0.068 **	0.011 **	1.000					
T2		-0.192 **	0.258 **	-0.014 **	-0.075 **	-0.174 **	0.040 **	-0.015 **	-0.358 **	-0.137 **	0.189 **	0.102 **	0.112 **	0.174 **	0.272 **	0.263 **	0.427 **	1.000				
T3		0.238 **	0.124 **	-0.442 **	0.044 **	0.283 **	0.174 **	-0.031 **	-0.041 **	0.259 **	0.058 **	0.364 **	0.025 **	-0.307 **	0.028 **	0.036 **	-0.180 **	0.223 **	1.000			
T4		-0.169 **	-0.116 **	0.087 **	0.170 **	0.268 **	-0.237 **	0.353 **	0.267 **	0.121 **	0.133 **	-0.060 **	0.047 **	0.172 **	0.090 **	0.059 **	-0.145 **	0.412 **	0.049 **	1.000		
T5		-0.196 **	0.228 **	0.325 **	-0.370 **	-0.348 **	-0.444 **	0.293 **	-0.206 **	-0.519 **	-0.151 **	-0.168 **	-0.083 **	0.051 **	0.263 **	0.503 **	0.305 **	0.310 **	-0.519 **	0.076 **	1.000	
T6		-0.207 **	0.046 **	0.217 **	-0.287 **	0.267 **	0.302 **	-0.220 **	-0.044 **	0.153 **	-0.170 **	0.041 **	-0.339 **	0.308 **	-0.086 **	-0.050 **	0.499 **	0.456 **	0.128 **	0.316 **	0.101 **	1.000
	C																D					E

Where, Morphometric traits are included in the area DEFD: T1= Plant height, T2= Number of branches, T3=Root length, T4=Plant fresh weight, T5= Stem circumference, T6= Branch length; Chemometric traits are included in the area ABFA : F1=Eucalyptol, F2= endo-Borneol, F3= 3-Carene, F4= Camphene, F5= alpha.-Pinene, F6= p-Cymene, F7= o-Cymene, F8= Cyclobutane, 1,2-dicyclopropyl-, F9= Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-, F10= Linalool, F11= D-Limonene, F12= (+)-2-Bornanone, F13= .alpha.-Phellandrene, F14= beta.-Phellandrene, F15= Linalyl acetate.; Area BCDFB includes correlation coefficients encompassing morphometric and chemometric traits;

*** = $p < 0.01$; Gradual transformation of green colour to red colour indicates highly positive significant values to highly negative significant values respectively.*

Figure 11: Correlation between twenty one traits of *L. angustifolia* (somaclones).

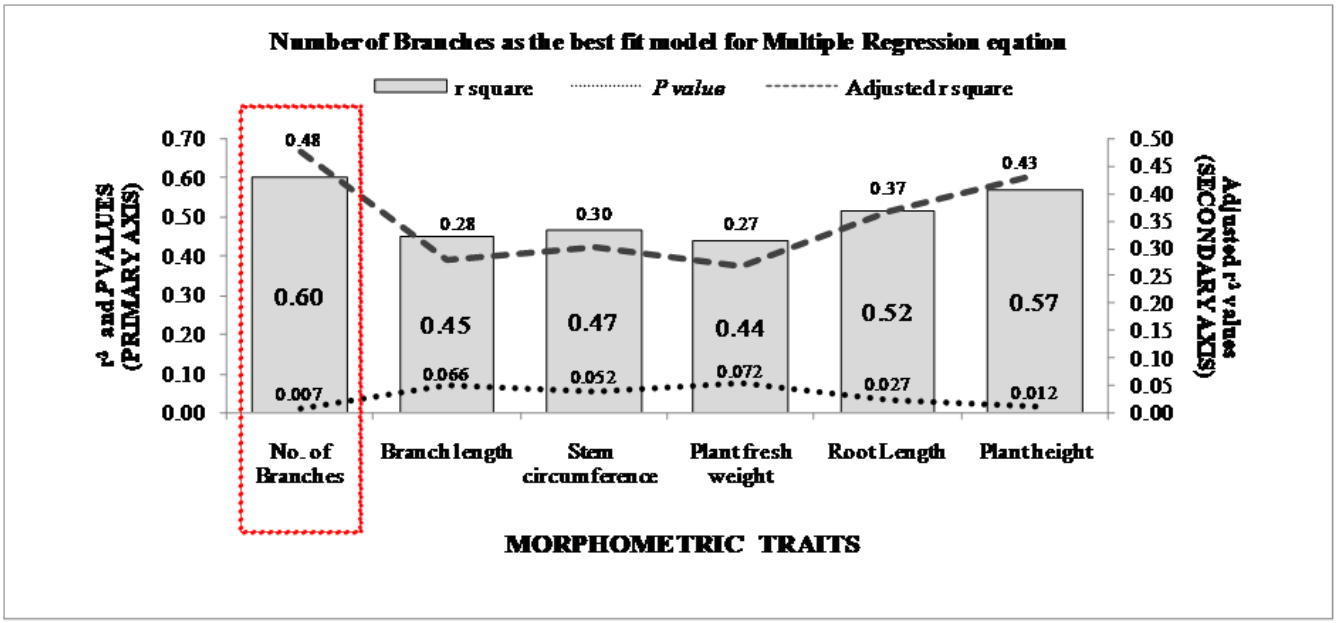
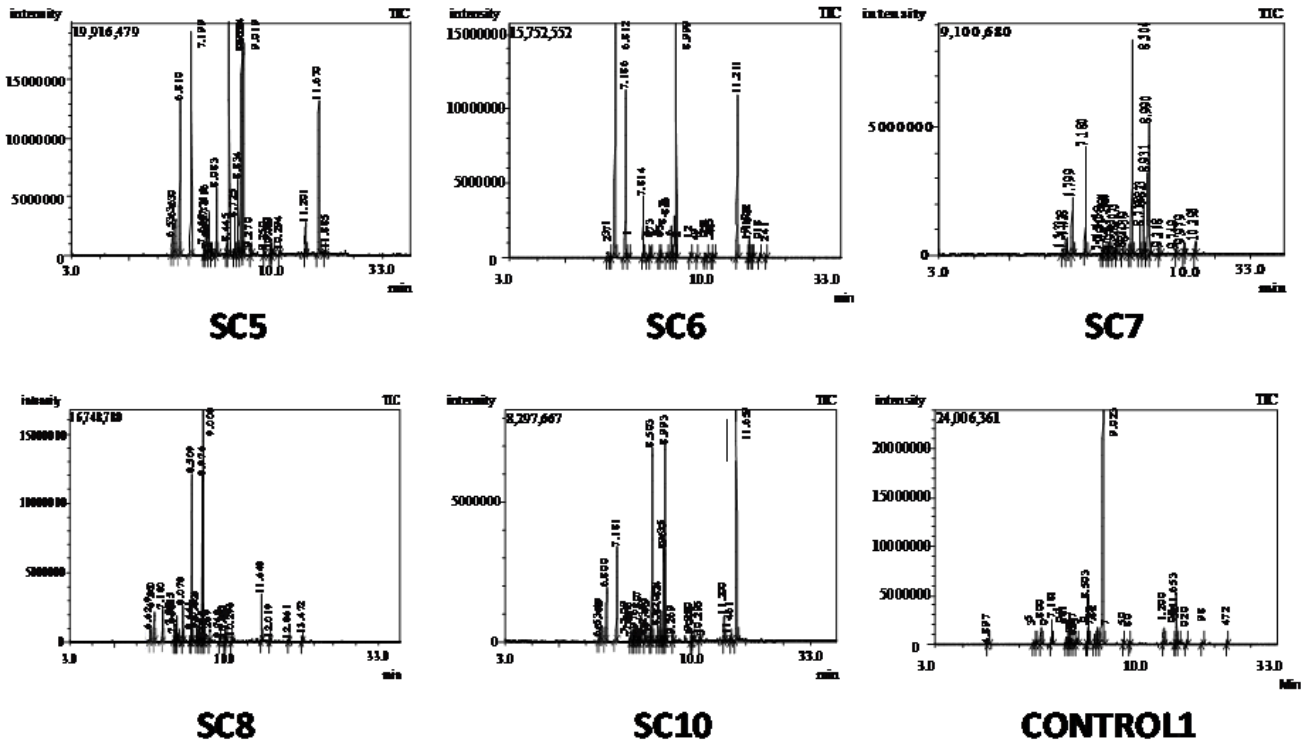


Figure 12: Regression analysis with six morphometric traits and identification of the best regression model.

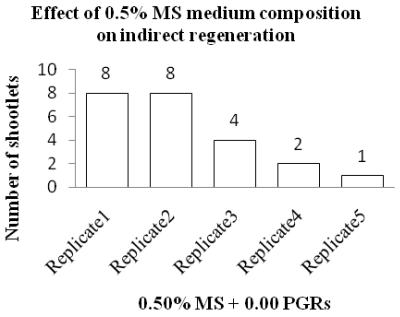
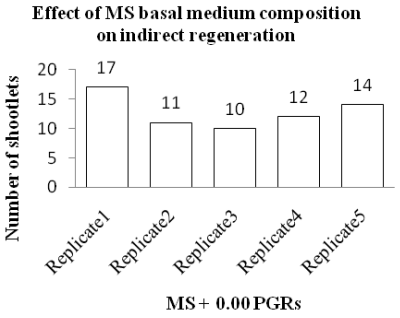
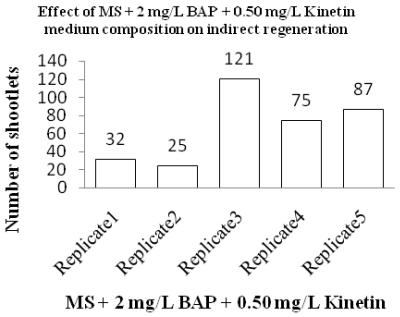


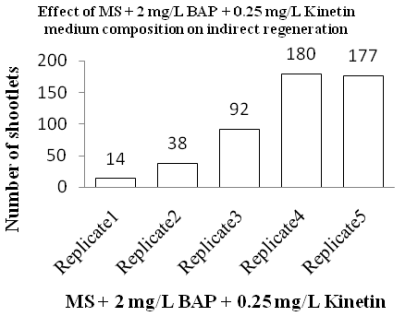
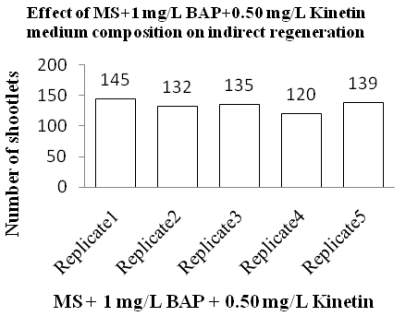
Highest concentration of essential oil components present in leaves of some treatments(SC5-8) and control1.					
Control1	SC5	SC6	SC7	SC8	SC10
Eucalyptol RT: 9.023 Peak# 15	*α-pinene* RT : 6.810 Peak# 3	*Camphene* RT : 7.186 Peak# 4	*Linalool* RT :10.295 Peak# 21	*Linalyl acetate* RT : 12.861 Peak# 22	*α-phellandrene* RT : 8.439 Peak# 11
	o-cymene RT : 8.725 Peak# 12 RT : 8.834 Peak#13	*(+)-2-Bornanone* RT : 11.211 Peak# 21			*Cyclobutane, 1,2-dicyclopropyl-* RT : 8.935 Peak# 15
	D-Limonene RT : 8.966 Peak# 14				

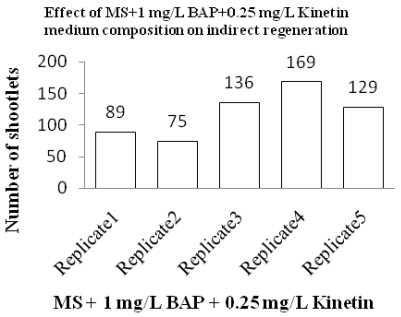
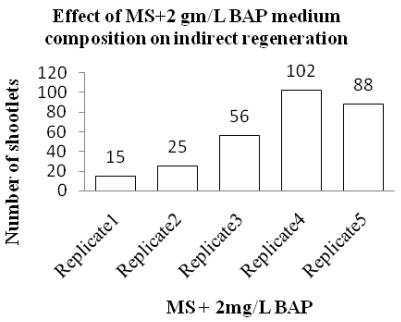
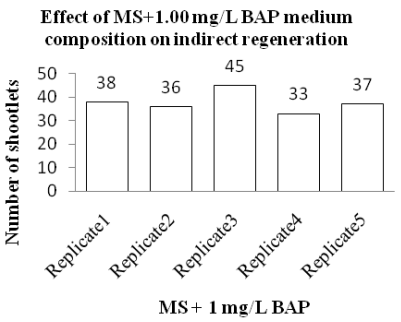
Figure 13: HS-GCMS chromatograms of somaclones(SC) 5,6,7,8,10 and control1 of *L. angustifolia* representing variation in essential oil profile.

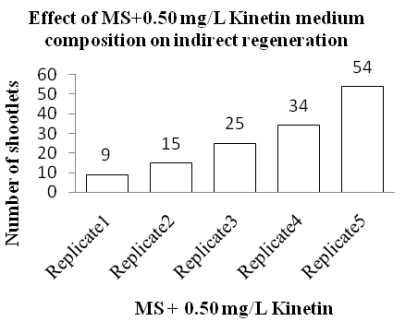
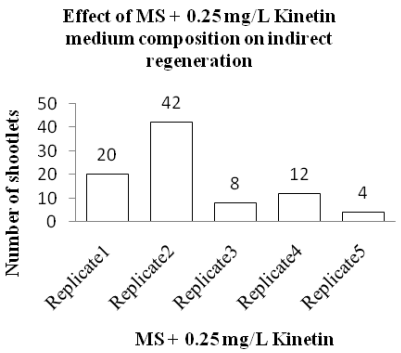
Figure 14: The resultant putative somaclonal variants (SC5, SC6 and SC10) and Control1 of *L. angustifolia* in potted and exposed condition.

Supplementary Table : Caulogenesis

Sl No	Particulars	Details		Histogram
1	Media composition	0.50% MS		
	PGR	Nil		
	Culture duration (weeks)	03		
	Mean of regeneration	4.60±3.29		
	Characteristics of the culture	a. callus ceased to grow, browning of callus. b. frequency of regeneration is least. c. survival rate of regenerents is poorest. d. yellow leaflets with internodal elongation in shootlets.		
2	Media composition	MS		
	PGR	Nil		
	Culture duration (weeks)	03		
	Mean of regeneration	12.80±2.77		
	Characteristics of the culture	a. callus giving rise to healthy shootlets. b. frequency of regeneration is poor. c. survival rate of regenerents considerable. d. green leaflets with healthy shootlets.		
3	Media composition	MS		
	PGR	2.00 mg/l BAP + 0.50 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	68.00±39.89		

	Characteristics of the culture	a. callus giving rise to healthy shootlets. b. frequency of regeneration is good. c. survival rate of regenerents more considerable. d. green leaflets with healthy shootlets. e. leaflets densely arranged in lower part of the shootlets and internodal elongation in shootlets.		
4	Media composition	MS		
	PGR	2.00 mg/l BAP + 0.25 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	100.20±76.86		
	Characteristics of the culture	a. callus giving rise to healthy shootlets. b. abundant healthy regenerents. c. primary branching observed. d. green leaflets with healthy shootlets. e. leaflets densely arranged in lower part of the shootlets and internodal elongation in shootlets.		
5	Media composition	MS		
	PGR	1.00 mg/l BAP + 0.50 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	134.20±9.31		
	Characteristics of the culture	a. callus giving rise to very healthy shootlets. b. most abundant healthy regenerents. c. primary branching observed more. d. green leaflets with healthy shootlets. e. no internodal elongation in shootlets.		

6	Media composition	MS		
	PGR	1.00 mg/l BAP + 0.25 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	119.60±37.83		
	Characteristics of the culture	a. callus giving rise to very healthy shootlets. b. abundant healthy regenerents. c. primary branching less frequent. d. green leaflets with healthy shootlets. e. leaflets arranged normally over shootlets with no internodal elongation in the later.		
7	Media composition	MS		
	PGR	2.00 mg/l BAP		
	Culture duration (weeks)	03		
	Mean of regeneration	57.20±38.00		
	Characteristics of the culture	a. callus giving rise to very healthy shootlets. b. abundant regenerents with delayed response c. green leaflets with healthy shootlets. d. leaflets arranged normally over shootlets with no internodal elongation in the later.		
8	Media composition	MS		
	PGR	1.00 mg/l BAP		
	Culture duration (weeks)	03		
	Mean of regeneration	37.80±4.44		

	Characteristics of the culture	a. callus giving rise to very healthy shootlets. b. abundant regenerents with delayed response c. green leaflets with healthy shootlets. d. leaflets arranged normally over shootlets with no internodal elongation in the later.		
9	Media composition	MS		
	PGR	0.50 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	27.40±17.67		
	Characteristics of the culture	a. callus giving rise to less healthy shootlets. b. leafy regenerents on callus are yellowish. c. green leaflets with slender shootlets. d. leaflets arranged norm- ally over shootlets with no internodal elon- gation in the later.		
10	Media composition	MS		
	PGR	0.25 mg/l Kinetin		
	Culture duration (weeks)	03		
	Mean of regeneration	17.20±15.07		
	Characteristics of the culture	a. callus giving rise to less healthy shootlets. b. bfrequency of regenerated shootlets are lesser. c. leafy regenerents on callus are more yellowish. d. green leaflets with slender shootlets. e. leaflets arranged normally over shootlets with no internodal elongation in the later.		

Solutions of Negative Pell's Equation Invoving Pierpont Primes, Consecutive Good and Proth Primes

J. Kannan, Manju Somanath & K. Raja

Madurai Kamaraj University

ABSTRACT

Many researchers have been devoted to nding the solutions (η, ζ) in the set of non-negative integers, of Diophantine equation (Pell Equation) of the type $\eta^2 = D\zeta^2 \pm \alpha$ where the value α is xed positive integers. in this article, we look for non-trivial integer solutions to the negative Pell equation$x^2 = \phi y^2 - {}^t$, where ϕ, ψ are the Pierpont Primes, Consecutive Good and Proth Primes, here $t \in N$, for the different choices of t particular by *(i) t = 1, (ii) t = 3, (iii) t = 5,(iv) t = 2k, (v) t = 2k + 5,for all k 2* N.

Keywords: pell equation, integral solutions, diophantine equations, pierpont primes, consecutive good prime, consecutive proth primes, pythagorean primes, brahma gupta lemma.

Classification: DDC Code: 511.6 LCC Code: QA241

Language: English

London Journals Press

LJP Copyright ID: 925675
Print ISSN: 2631-8490
Online ISSN: 2631-8504

London Journal of Research in Science: Natural and Formal

Volume 22 | Issue 7 | Compilation 1.0

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Many researchers have been devoted to nding the solutions (η, ζ) in the set of non-negative integers, of Diophantine equation (Pell Equation) of the type $\eta^2 = D\zeta^2 \pm \alpha$ where the value α is xed positive integers. in this article, we look for non-trivial integer solutions to the negative Pell equation $x^2 = \phi y^2 - \psi^t$, where ϕ, ψ are the Pierpont Primes, Consecutive Good and Proth Primes, here $t \in N$, for the different choices of t particular by *(i) t = 1, (ii) t = 3,(iii) t = 5,(iv) t = 2k, (v) t = 2k + 5,for all k 2* N.

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I. INTRODUCTION

In this paper, Negative Pell equations are considered for their integral solutions in each of the three sections 4.1 to 4.3 as follows.

Pells equation (also called Pell-Fermats equation) is any Diophantine equation $x^2 - dy^2 = 1$, where d is a given positive non-square integer and integer solutions are sought for x and y. In Cartesian coordinates, the equation has the form of a hyperbola; solutions occur whenever the curve passes through a point whose x and y coordinates are both integers, such as the trivial solution with x = 1 and y = 0 Joseph Loius proved that, as long as n is not a perfect square, Pells equation has infinitely many distinct integer solutions. These solutions may be used to accurately approximately the square root of n by rational number of the form $\frac{x}{y}$.

The negative Pell equation is given by $x^2 - dy^2 = -1$. It has also been extensively studied; it can be solved by the same method of continued fractions and will have solutions if and only if the period of the continued fraction has odd length. However it is not known which roots have been odd period lengths and therefore not known when the negative Pell equation is solvable. A necessary (but not sufficient) condition for solvability is that n is not divisible by 4 or by a prime of form $4k + 3$. Thus, for example, $x^2 - 3ny^2 = -1$ is never solvable, but $x^2 - 5ny^2 = -1$ may be.

Theorem 1.1. *If (x_1, y_1) is the fundamental solution of $x^2 - dy^2 = 1$. Then every positive solution of the equation is given by (x_n, y_n), where x_n and y_n are the integers determined from*

$$x_n + y_n\sqrt{d} = (x_1 + y_1\sqrt{d})^n, \ n = 1, 2, 3, \cdots$$

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Proof. In anticipation of a contradiction, let us suppose that there exists a positive solution u, v that is not obtainable by the formula $(x_1 + y_1\sqrt{d})^n$. Because $x_1 + y_1\sqrt{d} > 1$, the powers of $x_1 + y_1\sqrt{d}$ become arbitrarily large; this means that $u + v\sqrt{d}$ must lie between two consecutive powers of $x_1 + y_1\sqrt{d}$, say.

$$(x_1 + y_1\sqrt{d})^n < u + v\sqrt{d} < (x_1 + y_1\sqrt{d})^{n+1}$$

or, to phrase it in different terms,

$$x_n + y_n\sqrt{d} < u + v\sqrt{d} < (x_n + y_n\sqrt{d})(x_1 + y_1\sqrt{d})$$

On multiplying this inequality by the positive number $x_n - y_n\sqrt{d}$ and nothing that $x_n^2 - dy_n^2 = 1$, we are led to

$$1 < (x_n - y_n\sqrt{\bar{d}})(u + v\sqrt{\bar{d}}) < x_1 + y_1\sqrt{\bar{d}}$$

Next define the integers r and s by $r + s\sqrt{d} = (x_n - y_n\sqrt{d})(u + v\sqrt{d})$; that is, let

$$r = x_n u - y_n vd$$
$$s = x_n v - y_n u$$

An easy calculation reveals that

$$\begin{aligned} r^2 - ds^2 &= (x_n^2 - dy_n^2)(u^2 - dv^2) \\ &= 1 \end{aligned}$$

and therefore r, s is a solution of $x^2 - dy^2 = 1$ satisfying

$$1 < r + s\sqrt{d} < x_1 + y_1\sqrt{d}$$

Completion of the proof requires us to show that the pair (r, s) is a positive solution. Because $1 < r + s\sqrt{d}$ and $(r + s\sqrt{d})(r - s\sqrt{d}) = 1$, we find that $0 < r - s\sqrt{d} < 1$. In consequence

$$2r = (r + s\sqrt{d}) + (r - s\sqrt{d}) > 1 + 0 > 0$$

$$2s\sqrt{d} = (r + s\sqrt{d}) + (r - s\sqrt{d}) > 1 - 1 = 0$$

which makes both r and s positive. The upshot is that because, (x_1, y_1) is the fundamental solution of $x^2 - dy^2 = 1$, we must have $x_1 < r$ and $y_1 < s$; but then $x_1 + y_1\sqrt{d} < r + s\sqrt{d}$, violating an earlier inequality. This contradiction ends our argument.

Theorem 1.2. *Let p be a prime. The negative Pells equation $x^2 - py^2 = -1$ is solvable if and only if $p = 2$ or $p \equiv 1(mod 4)$.*

Testing the solubility of the negative Pell equation:

Suppose D is a positive integer, not a perfect square. Then the negative Pell equation $x^2 - Dy^2 = -1$ is soluble if and only if D is expressible as $D = a^2 + b^2, \gcd(a, b) = 1$. a and b positive, b odd and the Diophantine equation $-bV^2 + 2aVW + bW^2 = 1$ has a solution(The Case of solubility occurs for exactly one such (a, b)).

The Algorithm:

(1) Find all expression of D a sum of two relatively prime squares using Cornacchia's method. If none, exist - the negative Pell equation is not soluble.
(2) For each representation $D = a^2 + b^2, \gcd(a, b) = 1$, a and b positive, b odd, test the solubility of $-bV^2 + 2aWV + bW^2 = 1$ using the Lagrange - Matthews algorithm. If solution exist - the negative Pell equation is soluble.
(3) If each representation yields no solution, then the negative Pell equation is insoluble.

II. SOLUTIONS OF PELL'S EQUATION INVOLVING PIERPONT PRIMES

In this section, concerns with the Pell equation $x^2 = 73y^2 - 3^t, t \in \mathbb{N}$, and infinitely many positive integer solutions are obtained for the choices of t given by $(i)t = 1,\ (ii)t = 3,\ (iii)t = 5, (iv)t = 2k$ and $(v)t = 2k + 5, k \in \mathbb{N}$.

A Pierpont prime is a prime number of the form $2^u 3^v + 1$ for some nonnegative integers u and v. Here using Pierpont primes 3 and 73 we form a Pell's equation $x^2 = 73y^2 - 3^t, t \in \mathbb{N}$ and search for its non-trivial integer solutions. A few interesting relations among the solutions are presented. Further recurrence relations on the solutions are derived.

Choice 1: $t = 1$

The Pell equation is

$$x^2 = 73y^2 - 3 \tag{1}$$

Let (x_0, y_0) be the initial solution of (1) . Then $x_0 = 17;\ y_0 = 2$.
To find the other solutions of (1), consider the Pell equation

$$x^2 = 73y^2 + 1$$

whose initial solution $(\tilde{x_n}, \tilde{y_n})$ is given by

$$\tilde{x_n} = \frac{1}{2} f_n$$
$$\tilde{y_n} = \frac{1}{2\sqrt{73}} g_n$$

where

$$f_n = \frac{1}{2}[(2281249 + 267000\sqrt{73})^{n-1} + (2281249 - 267000\sqrt{73})^{n+1})]$$
$$g_n = \frac{1}{2\sqrt{73}}[(2281249 + 267000\sqrt{73})^{n-1} - (2281249 - 267000\sqrt{73})^{n+1})]$$

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions to (1) are obtained as

$$x_{n+1} = \frac{1}{2}[17 f_n + 2\sqrt{73} g_n] \tag{2}$$

$$y_{n+1} = \frac{1}{2\sqrt{73}}[2\sqrt{73} f_n + 17 g_n] \tag{3}$$

The recurrence relation satisfied by the solutions of (1) are given by

$$x_{n+2} - 534000 x_{n+1} + x_n = 0$$
$$y_{n+2} - 534000 y_{n+1} + y_n = 0$$

Choice 2: $t = 3$

The Pell equation is

$$x^2 = 73y^2 - 3^3 \tag{4}$$

with the initial solution $x_0 = 51;\ y_0 = 6$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[51f_n + 6\sqrt{73}g_n]$$
$$y_{n+1} = \frac{1}{2\sqrt{73}}[6\sqrt{73}f_n + 51g_n]$$

The recurrence relation satisfied by the solutions of (4) are given by

$$x_{n+2} - 534000x_{n+1} + x_n = 0$$
$$y_{n+2} - 534000y_{n+1} + y_n = 0$$

Choice 3: $t = 5$

The Pell equation is

$$x^2 = 73y^2 - 3^5 \tag{5}$$

with the initial solution $x_0 = 153;\ y_0 = 18$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[153f_n + 18\sqrt{73}g_n]$$
$$y_{n+1} = \frac{1}{2\sqrt{73}}[18\sqrt{73}f_n + 153g_n].$$

The recurrence relation satisfied by the solutions of (5) are given by

$$x_{n+2} - 534000x_{n+1} + x_n = 0$$
$$y_{n+2} - 534000y_{n+1} + y_n = 0$$

Choice 4: $t = 2k,\ k \in \mathbb{N}$

The Pell equation is

$$x^2 = 73y^2 - 3^{2k} \tag{6}$$

with the initial solution $x_0 = 1068(3^k);\ y_0 = 125(3^k)$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{3^k}{2}[1068f_n + 125\sqrt{73}g_n]$$
$$y_{n+1} = \frac{3^k}{2\sqrt{73}}[125\sqrt{73}f_n + 1068g_n]$$

The recurrence relation satisfied by the solutions of (6) are given by

$$x_{n+2} - 534000x_{n+1} + x_n = 0$$
$$y_{n+2} - 534000y_{n+1} + y_n = 0$$

Choice 5: $t = 2k + 5, k \in \mathbb{N}$

The Pell equation is

$$x^2 = 73y^2 - 3^{2k+5} \tag{7}$$

with the initial solution $x_0 = 5861(3^{k-1});\ y_0 = 686(3^{k-1})$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{3^{k-1}}{2}[5861f_n + 686\sqrt{73}g_n]$$
$$y_{n+1} = \frac{3^{k-1}}{2\sqrt{73}}[686\sqrt{73}f_n + 5861g_n]$$

The recurrence relations satisfied by the solutions of (7) are given by

$$x_{n+2} - 534000x_{n+1} + x_n = 0$$
$$y_{n+2} - 534000y_{n+1} + y_n = 0$$

III. INTEGRAL SOLUTIONS OF NEGATIVE PELL'S EQUATION INVOLVING CONSECUTIVE GOOD Primes $x^2 = 41y2 - 37^t$

Let $d \neq 1$ be a positive non - square integer and N be any fixed positive integer. Then the equation $x^2 - dy^2 = \pm N$ is known as Pell's equation named after the famous Mathematician John Pell.

A *good prime* is a prime number whose square is greater than the product of any two primes at the same number of positions before and after it in the sequence of primes.
That is, a good prime satisfies the inequality $p_n^2 > p_{n-i} \times p_{n+i}$, for all $1 \leq i \leq n-1$ where p_n is the n^{th} prime.

In this section, we fix d and N to be two consecutive good primes 41 and 37 and search for non - trivial integer solution to the equation $x^2 = 41y^2 - 37^t, t \in \mathbb{N}$ for the different choices of t given by $(i)t = 1,\ (ii)t = 3,\ (iii)t = 5,\ (iv)t = 2k,\ \forall k \in \mathbb{N}$ and $(v)t = 2k+5,\ \forall k \in \mathbb{N}$. Further, recurrence relation on the solutions are obtained.

By testing the solubility of the negative Pell equation, solving $x^2 + y^2 = 41$ we get $(x, y) = (5, 4)$. Number of positive primitive solutions with $x \geq y$ is 1.

(1): Testing $(a, b) = (4, 5)$.
(2): $-bV^2 + 2aVW + bW^2 = 1$ has a solution $(V, W) = (2, 1)$.

So $x^2 - 41y^2 = -1$ is solvable.

Choice 1: $t = 1$
The Pell equation is

$$x^2 = 41y^2 - 37 \tag{8}$$

with the initial solution $x_0 = 2;\ y_0 = 1$.

To find the other solutions, consider the more general Pell equation $x^2 = 41y^2 + 1$ whose general solution $(\tilde{x_n}, \tilde{y_n})$ is given by

$$\tilde{x_n} = \frac{1}{2}f_n;\ \tilde{y_n} = \frac{1}{2\sqrt{41}}g_n.$$

where $f_n = (2049 + 320\sqrt{41})^{n+1} + (2049 - 320\sqrt{41})^{n+1}$ and $g_n = (2049 + 320\sqrt{41})^{n+1} - (2049 - 320\sqrt{41})^{n+1},\ n = 0, 1, \cdots$.
Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[2f_n + \sqrt{41}g_n]$$
$$y_{n+1} = \frac{1}{2\sqrt{41}}[\sqrt{41}f_n + 2g_n]$$

The recurrence relations satisfied by the solutions of (8) are given by

$$x_{n+2} - 4098x_{n+1} + x_n = 0$$
$$y_{n+2} - 4098y_{n+1} + y_n = 0$$

Choice 2: $t = 3$
The Pell equation is

$$x^2 = 41y^2 - 37^3 \tag{9}$$

with the initial solution $x_0 = 254;\ y_0 = 53$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as $x_{n+1} = \frac{1}{2}[254f_n + 53\sqrt{41}g_n]$ and $y_{n+1} = \frac{1}{2\sqrt{41}}[53\sqrt{41}f_n + 254g_n]$.

The recurrence relations satisfied by the solutions of (9) are given by

$$x_{n+2} - 4098x_{n+1} + x_n = 0$$
$$y_{n+2} - 4098y_{n+1} + y_n = 0$$

Choice 3: $t = 5$
The Pell equation is

$$x^2 = 41y^2 - 37^5 \tag{10}$$

with the initial solution $x_0 = 9398;\ y_0 = 1961$.
Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[9398f_n + 1961\sqrt{41}g_n]$$
$$y_{n+1} = \frac{1}{2\sqrt{41}}[1961\sqrt{41}f_n + 9398g_n]$$

The recurrence relations satisfied by the solutions of (10) are given by

$$x_{n+2} - 4098x_{n+1} + x_n = 0$$
$$y_{n+2} - 4098y_{n+1} + y_n = 0$$

Choice 4: $t = 2k,\ k \in \mathbb{N}$

The Pell equation is

$$x^2 = 41y^2 - 37^{2k} \tag{11}$$

with the initial solution $x_0 = 32(37^k);\ y_0 = 5(37^k)$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non - zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{37^k}{2}[32f_n + 5\sqrt{41}g_n]$$
$$y_{n+1} = \frac{37^k}{2\sqrt{41}}[5\sqrt{41}f_n + 32g_n].$$

The recurrence relations satisfied by the solutions of (11) are given by

$$x_{n+2} - 4098x_{n+1} + x_n = 0$$
$$y_{n+2} - 4098y_{n+1} + y_n = 0$$

Choice 5: $t = 2k + 5, k \in \mathbb{N}$
The Pell equation is

$$x^2 = 41y^2 - 37^{2k+5} \tag{12}$$

with the initial solution $x_0 = 347726(37^{k-1});\ y_0 = 72557(37^{k-1})$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{37^{k-1}}{2}[347726f_n + 72557\sqrt{41}g_n]$$

$$y_{n+1} = \frac{37^{k-1}}{2\sqrt{41}}[72557\sqrt{41}f_n + 347726g_n]$$

The recurrence relations satisfied by the solutions of (12) are given by

$$x_{n+2} - 4098x_{n+1} + x_n = 0$$
$$y_{n+2} - 4098y_{n+1} + y_n = 0$$

IV. OBSERVATION ON NEGATIVE PELL'S EQUATION INVOLVING CONSECUTIVE PROTH PRIMES $x^2 = 13y^2 - 17^t$

In this section, we fix d and N to be two consecutive proth primes 13 and 17 and search for non - trivial integer solution to the equation $x^2 = 13y^2 - 17^t, t \in \mathbb{N}$ for the different choices of t given by $(i)t = 1,\ (ii)t = 3,\ (iii)t = 5,\ (iv)t = 2k,\ \forall k \in \mathbb{N}$ and $(v)t = 2k + 5,\ \forall k \in \mathbb{N}$. Further, recurrence relation on the solutions are obtained.

By testing the solubility of the negative Pell equation, solving $x^2+y^2 = 13$ we have $(x, y) = (3, 2)$. Number of positive primitive solutions with $x \geq y$ is 1.

(1): Testing $(a, b) = (2, 3)$.
(2): $-bV^2 + 2aVW + bW^2 = 1$ has a solution $(V, W) = (71, 38)$.
So $x^2 - 13y^2 = -1$ is solvable.

Choice 1: $t = 1$

The Pell equation is

$$x^2 = 13y^2 - 17 \tag{13}$$

with the initial solution $x_0 = 10;\ y_0 = 3$.

To find the other solutions, consider the more general Pell equation $x^2 = 13y^2 + 1$ whose general solution $(\tilde{x_n}, \tilde{y_n})$ is given by

$$\tilde{x_n} = \frac{1}{2}f_n;\ \tilde{y_n} = \frac{1}{2\sqrt{13}}g_n.$$

where $f_n = (649 + 180\sqrt{13})^{n+1} + (649 - 180\sqrt{13})^{n+1}$ and $g_n = (649 + 180\sqrt{13})^{n+1} - (649 - 180\sqrt{13})^{n+1},\ n = 0, 1, \cdots$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[10f_n + 3\sqrt{13}g_n]$$

$$y_{n+1} = \frac{1}{2\sqrt{13}}[3\sqrt{13}f_n + 10g_n]$$

The recurrence relations satisfied by the solutions of (13) are given by

$$x_{n+2} - 1298x_{n+1} + x_n = 0$$
$$y_{n+2} - 1298y_{n+1} + y_n = 0$$

Choice 2: $t = 3$
The Pell equation is

$$x^2 = 13y^2 - 17^3 \tag{14}$$

with the initial solution $x_0 = 350;\ y_0 = 99$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as $x_{n+1} = \frac{1}{2}[350f_n + 99\sqrt{13}g_n]$ and $y_{n+1} = \frac{1}{2\sqrt{13}}[99\sqrt{13}f_n + 350g_n]$.

The recurrence relations satisfied by the solutions of (14) are given by

$$x_{n+2} - 1298x_{n+1} + x_n = 0$$
$$y_{n+2} - 1298y_{n+1} + y_n = 0$$

Choice 3: $t = 5$
The Pell equation is

$$x^2 = 13y^2 - 17^5 \tag{15}$$

with the initial solution $x_0 = 1270;\ y_0 = 483$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{1}{2}[1270f_n + 483\sqrt{13}g_n]$$
$$y_{n+1} = \frac{1}{2\sqrt{13}}[483\sqrt{13}f_n + 1270g_n]$$

The recurrence relations satisfied by the solutions of (15) are given by

$$x_{n+2} - 1298x_{n+1} + x_n = 0$$
$$y_{n+2} - 1298y_{n+1} + y_n = 0$$

Choice 4: $t = 2k,\ k \in \mathbb{N}$
The Pell equation is

$$x^2 = 13y^2 - 17^{2k} \tag{16}$$

with the initial solution $x_0 = 18(17^k);\ y_0 = 5(17^k)$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non - zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{17^k}{2}[18f_n + 5\sqrt{13}g_n]$$
$$y_{n+1} = \frac{17^k}{2\sqrt{13}}[5\sqrt{13}f_n + 18g_n].$$

The recurrence relations satisfied by the solutions of (16) are given by

$$x_{n+2} - 1298x_{n+1} + x_n = 0$$
$$y_{n+2} - 1298y_{n+1} + y_n = 0$$

Choice 5: $t = 2k + 5, k \in \mathbb{N}$

The Pell equation is

$$x^2 = 13y^2 - 17^{2k+5} \tag{17}$$

with the initial solution $x_0 = 203570(17^{k-1});\ y_0 = 56739(17^{k-1})$.

Applying Brahma Gupta lemma between (x_0, y_0) and $(\tilde{x_n}, \tilde{y_n})$, the sequence of non- zero distinct integer solutions are obtained as

$$x_{n+1} = \frac{17^{k-1}}{2}[203570f_n + 56739\sqrt{13}g_n]$$
$$y_{n+1} = \frac{17^{k-1}}{2\sqrt{13}}[56739\sqrt{13}f_n + 203570g_n]$$

The recurrence relations satisfied by the solutions of (17) are given by

$$x_{n+2} - 1298x_{n+1} + x_n = 0$$
$$y_{n+2} - 1298y_{n+1} + y_n = 0$$

V. CONCLUSION

Solving a Pells equation using the above method provides powerful tool for _nding solutions of equations of similar type. Neglecting any time consideration it is possible using current methods to determine the solvability of Pell like equation.

Author Contribution Statement: J. Kannan: Conceptualization, Writing - review, Editing, Supervision, Formal analysis & Data curation. Manju Somanath: Investigation, Writing - Original Draft. K. Raja: review, Data curation.

Funding: This research received no external funding.

Declaration of Competing Interest: The authors declare that they have no known competing_nancial interests or personal relationships that could have appeared to inuence the work reported in this paper. Data Availability Statement: No data were used to support this study.

ACKNOWLEDGMENTS

First authors thanks to Ayya Nadar Janaki Ammal College (Autonomous, a_liated to Madurai Kamaraj University, Madurai), Sivakasi, Tamil Nadu, India. Second and Third Authors thanks to National College (Autonomous, a_liated to Bharathidasan University, Trichy), Trichy, Tamil Nadu, India.

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