

**BIOPHYSICOCHEMICAL EVALUATION AND MICROPROPAGATION STUDIES ON NEEM
FOR BIODIESEL PRODUCTION**

Prithviraj Bhandare and G. R. Naik

Department of Biotechnology, Gulbarga University, Gulbarga - 585106, Karnataka, India.
prithvi.e87@gmail.com and grnaik123biotech@gmail.com

ABSTRACT: In this experiment the seed oils of 30 Neem (*Azadirachta indica*, A. juss) biotypes were screened and evaluated for their physio-chemical parameters for oil content, biodiesel yield, density, viscosity, iodine value, free fatty acid, saponification value, flash point and fire point which were estimated for selection of the elite neem biotype. The best shoot regeneration (60%-80%) was observed in Murashige and Skoog (MS) medium supplemented with naphthalene acetic acid NAA (0.2-0.4 mg/L) and benzyl amino purine BAP (0.2-0.4 mg/L). Root induction (80%) was successfully obtained in MS medium supplemented with IBA (0.05 mg/L) and IAA (0.05 mg/L). Acclimatization and hardening was quite successful with survival rate of 60%.

Key words: *Azadirachta indica*, oil, biodiesel, physio-chemical parameters, regeneration.

INTRODUCTION

The demand for energy supply in the world has increase tremendously due to increase in consumption rate. Report cited that the world non-renewable energy resources such as petroleum, gas, coal etc will soon be exhausted, hence there is need to act fast in other to start generating an alternative source of fuel that are renewable to replace the fossil fuel. The alternative fuel that is to replace the fossil fuel must be readily available, environmentally acceptable, economically competitive and technically feasible. Biodiesel is gaining increasing acceptance in the market as an environmental friendly alternative diesel fuel (Jidon Janaun et al. 2010). It is non-toxic, biodegradable, and free of sulphur or any carcinogenic compounds (Demirbas, 2007). The demand and cost of edible oils prevents its use in the production of biodiesel. So, a large variety of plants that produce non-edible oils are considered for biodiesel production. In India, there are several non-edible oil seed species such as *Jatropha curcas* (*Jatropha*), *Pongamia pinnata* (*Karanja*), *Azadirachta indica* (*Neem*), *Madhuca indica* (*Mahua*) etc., which could be utilized as a source for production of oil (Vuppaladadiyam et al. 2013) and can be grown in large scale on non-cropped marginal lands and waste lands. Fatty acid profiles of seed oils of 75 plant species having 30% or more fixed oil in their seed/kernel were examined (Mohibee, 2005), in which *Azadirachta indica*, *Calophyllum inophyllum*, *Jatropha curcas* and *Pongamia pinnata* were found to be most suitable for use as biodiesel and they meet the major specification of biodiesel standards of USA, Germany and European Standard Organization. The neem tree (*Azadirachta indica*) is a tropical evergreen with a wide adaptability, native to India and Burma, and been transplanted to Africa, the Middle East, South America, and Australia (Olugbenga Olufemi Awolu et al. 2013). It has been estimated that Indian neems bear about 3.5 million tons of kernels each year and that, in principle, about 700,000 tons can be recovered (Jeong GT, et al. 2004). About 34 tons of neem seeds were exported from India in 1990 (Awolu OO, et al. 2013). The neem plant is a fast growing plant with long productive life span of 150 to 200 years, its ability to survive on drought and poor soils at a very hot temperature of 44°C and a low temperature of up to 4°C has been reported (Aransiola EF et al, 2011), and its high oil content of 39.7 to 60% (Martín et al., 2010). A mature neem tree produces 30 to 50 kg fruit every year (Aransiola EF et al, 2011).

The prime aim of the current study is make it to be a useful renewable source for biodiesel production. To meet the demand in future, a large quantity of quality planting material will be needed. The micropropagation approach is considered to be the most appropriate viable alternative in tree improvement programmes.

Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods – like seeds or cuttings. Therefore, the current study also focuses on standardisation of micropropagation protocol for providing quality material for commercial cultivation of neem.

MATERIALS AND METHOD

Collection and Characterization of neem seeds

Thirty different neem biotypes were collected from the different parts of Bidar, Gulbarga, Raichur and Zaheerabad districts (Table 1). Biotypes were screened on the basis of physical appearance and oil-mass solidification. Seeds were inspected and manually cleaned to avoid foreign matter and physical properties such as seed weight, oil content and biodiesel content were determined.

Table 1. Characterisation of morphological and some chemical properties of neem plant

Provenance	No. of tress studied	Fruit weight of 10 seeds (gm)	Oil content (%)	Biodiesel Content (%)
Bidar	8	5.1	31.5	60
Gulbarga	5	6.8	43	85
Raichur	2	5.3	38	65
Zaheerabad	15	7.3	40.5	78

Neem seed preparation for oil extraction

The cleaned seeds were sun dried in the open, until the casing splits and sheds the seeds. The seeds were further dried in the oven at 60°C for 7hrs to a constant weight in order to reduce its moisture content, which was initially at about 5 to 7%. The separation of the shell from the nibs (cotyledon) was carried out using tray to blow away the cover in order to achieve very high yield. Mortar and pestle were used to crush the seeds into a paste (cake) in order to weaken or rupture the cell walls to release castor fat for extraction (Crentsil Kofi Bempah et al. 2011).

Neem oil extraction

150ml of normal hexane was poured into round bottom flask. 20 g of the sample was placed in the thimble and was inserted in the centre of the extractor. The Soxhlet was heated at 60°C. When the solvent was boiling, the vapour rises through the vertical tube into the condenser at the top. The liquid condensate drips into the filter paper thimble in the centre, which contains the solid sample to be extracted. The extract seeps through the pores of the thimble and fills the siphon tube, where it flows back down into the round bottom flask. This was allowed to continue for 30 minutes. It was then removed from the tube, dried in the oven, cooled in the desiccators and weighed again to determine the amount of oil extracted. Further extraction was carried out at 30 minutes interval until the sample weight at further extraction and previous weight becomes equal. The experiment was repeated for different weights of the sample, 35, 40 and 50 g. The weight of oil extracted was determined for each 30 minutes interval. At the end of the extraction, the resulting mixture (miscella) containing the oil was heated to recover solvent from the oil. Then, the following physicochemical parameters were estimated according to K. C. Verma et al. 2014.

Transesterification:

Biodiesel produced by transesterification by alkali process as it results in high purity and yield of the biodiesel product in a short time. Methyl esters of fatty acids were prepared by the procedure described in Singh, Kumar, and Sethi (2006). The yield of methyl esters was calculated using the following formula:

$$\text{Yield of methyl esters (\%)} = \frac{\text{Grams of methyl esters produced}}{\text{Grams of oil used in reaction}} \times 100$$

Density of Oil: Density of the sample is directly proportional to unsaturation and inversely to molecular weight. First, the empty cylinder was weighed, which was further filled with the sample and measured again.

Viscosity of oil: Viscosity is a measure of internal friction. It is an essential property which affects atomization of fuel and mixing of air and fuel in the combustion chamber. Viscosity of oil was measured using Ostwald's viscometer in which the sample was allowed to flow from the etched mark X–Y through the capillary of the viscometer. Viscosity was calculated as: $n_1/n_2 = d_1 t_1 / d_2 t_2$, where d_1 is the density of oil, d_2 the density of water, t_1 the time of flow for oil, t_2 the time of flow for water, n_1 the viscosity of oil, and n_2 the viscosity of water.

Free fatty acid content: It has a significant effect on the transesterification of glycerides with alcohol using catalyst. For determining free fatty acid (FFA) content, 25 mL of methanol was added to 1.5 g of each oil sample contained in the flask; the mixture was brought to boil in a water bath and then cooled. Two drops of phenolphthalein was added to the solution. 0.1 N NaOH was used to titrate the mixture with shaking for proper mixing.

$$\% \text{ FFA} = V \times 0.0282 \times 100 / \text{weight of sample}$$

Iodine value: The unsaturated fatty acid residues of the glycerides react with iodine, and the iodine value indicates the degree of unsaturation of the fatty acid residues of the glycerides. One gram of fat sample was taken in a stoppered bottle and 25 mL of Wij's solution was added to it. It was mixed properly and allowed to stand for 1 h. A blank was prepared with chloroform. With 50 mL distilled water the stopper and neck of the flask were rinsed thoroughly. Ten milliliters of KI solution was added to it. Then it was titrated with standard sodium thiosulphate (y) till it turned pale yellow. After that few drops of starch solution was added and titrated till blue colour disappears. The steps were repeated with a blank which did not contain any fat sample (x).

Saponification value: Saponification value indicates the presence of normal triglycerides, which can be used for production of liquid soap and shampoo. For determining saponification value, 1 g of oil sample was taken in different conical flasks and 3 mL of fat solvent was added to each flask. Twenty-five millilitres of ethanol potash was added and refluxed for 30 min with frequent shaking. After cooling, two drops of phenolphthalein indicator was added to each flask and titrated with 0.5 M HCl(x) with vigorous shaking without delay to get the end point. The step was repeated for a blank which did not contain oil sample (y).

$$\text{Saponification value} = 28.05 \times \text{Titre value (x-y)} / \text{weight of sample (g)}$$

Flash and fire point: Flash point is related to the safety requirement in the handling and storage of fuel; however, biodiesel falls under non-hazardous category. Flash point is the minimum temperature at which the oil vaporises, which when mixed with air forms an ignitable mixture and gives a momentary flash on application of a small pilot flame. The flash and fire points of the test fuels were measured using Pensky Martens apparatus. The sample was filled in the test cup up to the specified level and heated at a slow and constant rate of stirring for proper and uniform heating. The temperature was measured with the help of a thermometer of -10 to 400°C. At every 1°C temperature rise, the flame was directed into the cup through the opening provided at the top cover. The temperature at which flash was observed in the form of sound was recorded as the flash point of that sample. An extension of flash point is fire point, reflecting the condition at which vapour burns continuously for at least for 5 s.

Ash content: When organic compounds are decomposed at high temperature (500–600°C), the leftover residue is called ash. Ash content includes oxides and salts containing anions such as Cl⁻, SO₄²⁻ and other halides and cations such as Na⁺, K⁺, etc. For determining ash content, an electric muffle furnace was used. Five grams of oil sample was taken and placed in a dried and pre-weighed silver crucible. It was placed in the muffle furnace which resulted in burning away the polymer in an air atmosphere at a temperature of 500°C for 2 h and the crucible was weighed after it had been cooled to room temperature. The content was obtained using the equation given below:

$$A_s = \frac{W_a}{W_s} \times 100$$

Where A_s is the ash content (%), W_a is the weight of ash (g) and W_s is the weight of the sample (g).

Micropropagation study: The fresh plant parts namely young mature leaves, internodes, apical meristem were used as explants. They were brought to the lab and thoroughly washed by adding a drop of detergent Tween 20 and rinsed by keeping under running tap water for half an hour. They were surface sterilized with 0.1% sodium hypo chloride for 1 min followed by washing with distilled water 2-3 times (each wash is for 2 min) and wash with 70% ethanol for 1 min and wash with distilled water 2-3 times. Then washed with 0.1% HgCl₂ for 5 min followed by washing with sterile water for five times. The medium used for callus induction was Murashige and Skoog (MS) medium supplemented with naphthalene acetic acid (NAA: 0.1-0.5 mg/L), Kinetin (0.1-0.5 mg/L), 2,4-D (0.1-0.5 mg/L) and BAP in (0.1-0.5 mg/L). The percentages of callus induction frequency were recorded after 35 days. Proliferated calluses were sub-cultured for shooting (NAA: 0.5–1.0 mg/L+BAP: 0.5–5.0 mg/L) and finally for rooting in MS medium supplemented with IAA (0.01-0.05 mg/L) and IBA (0.01-0.05 mg/L).

RESULTS

The characteristic fuel properties such as relative density, viscosity, flash and fire points of neem oil and biodiesel were studied and compared with the Bureau of Indian Standards (BIS) and the fuel properties of diesel are presented in Table 2 and illustrated figures 1 to 11. The micropropagation results are presented in Table 3.

Table 2: Comparison of neem oil and biodiesel properties with BIS standards and diesel

Parameters	Neem oil				Neem biodiesel				BIS standards	Diesel
	Bidar	Gulbarga	Raichur	zaheerabad	Bidar	Gulbarga	Raichur	zaheerabad		
Density (gm/ml)	0.92	0.90	0.89	0.91	0.88	0.87	0.86	0.89	0.87-0.90	0.839
Viscosity (cST)	50.32	43.75	41.20	48.29	6.50	5.20	5.50	5.90	2.5-6.0	3.12
Iodine Value	81.28	77.53	78.89	80.64	--	--	--	--	--	--
Free fatty acid content	3.15	1.015	4.50	2.256	1.128	0.524	1.136	0.564	--	--
Saponification value	188.10	180.54	186.20	190.84	--	--	--	--	--	188-193
Flash point(oC)	--	--	--	--	165	150	158	160	>120	54.3
Fire point (oC)	--	--	--	--	182	158	165	180	>120	59.4
Ash Content	--	--	--	--	0.016	0.01	0.025	0.028	<0.02	0.0080

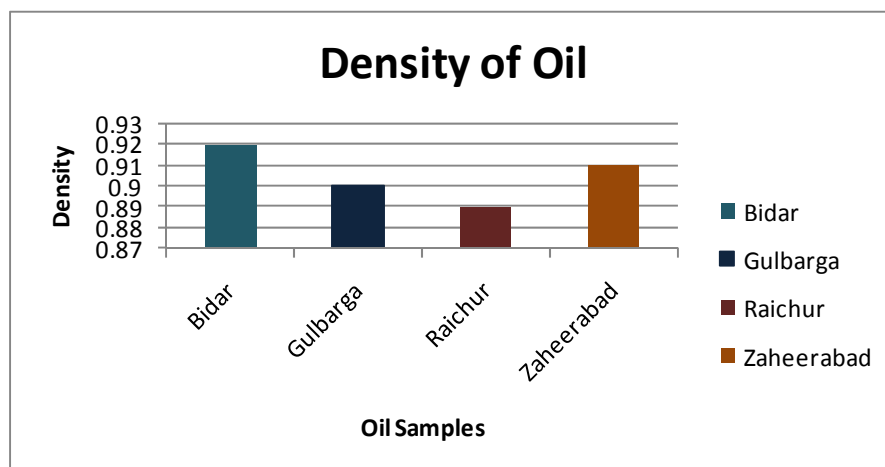


Figure 1: Density of Oil Samples from different area.

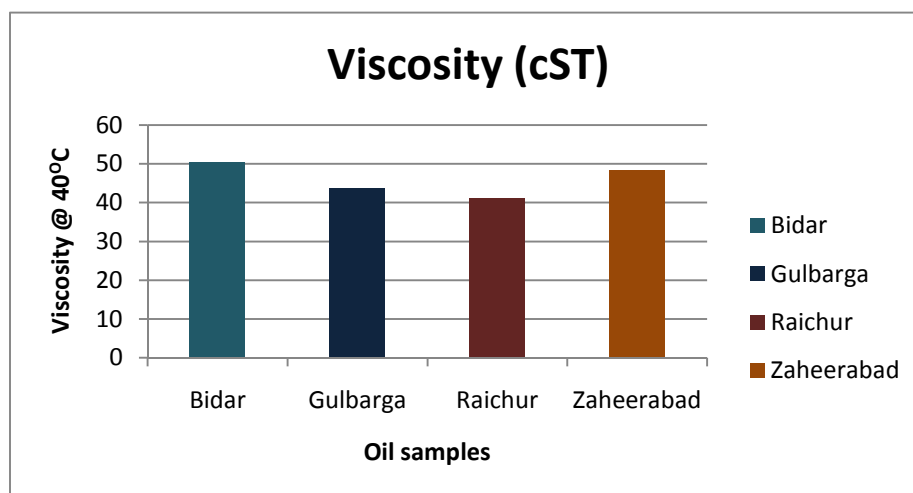


Figure 2: Viscosity of Oil Samples from different area.

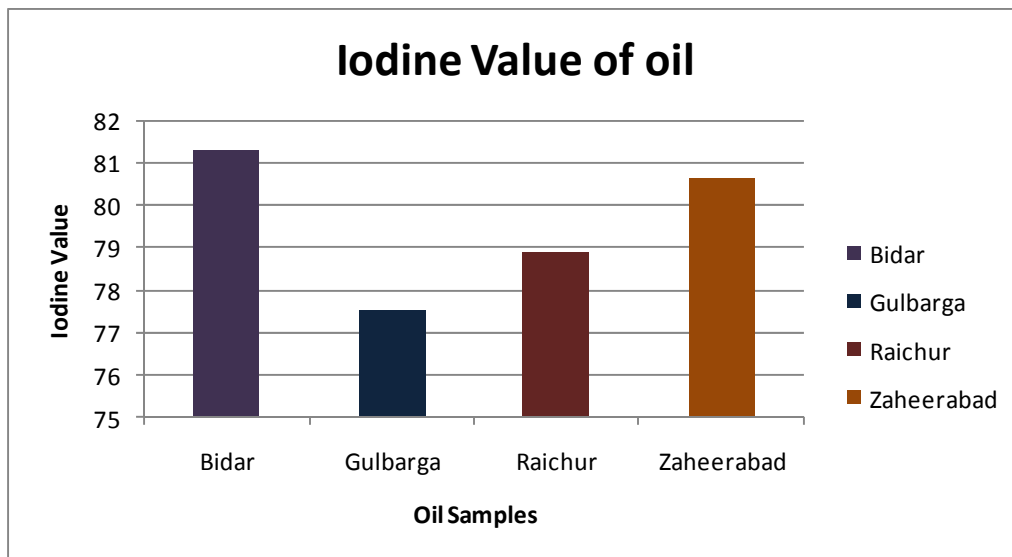


Figure 3: Iodine Value of Oil Samples from different area.

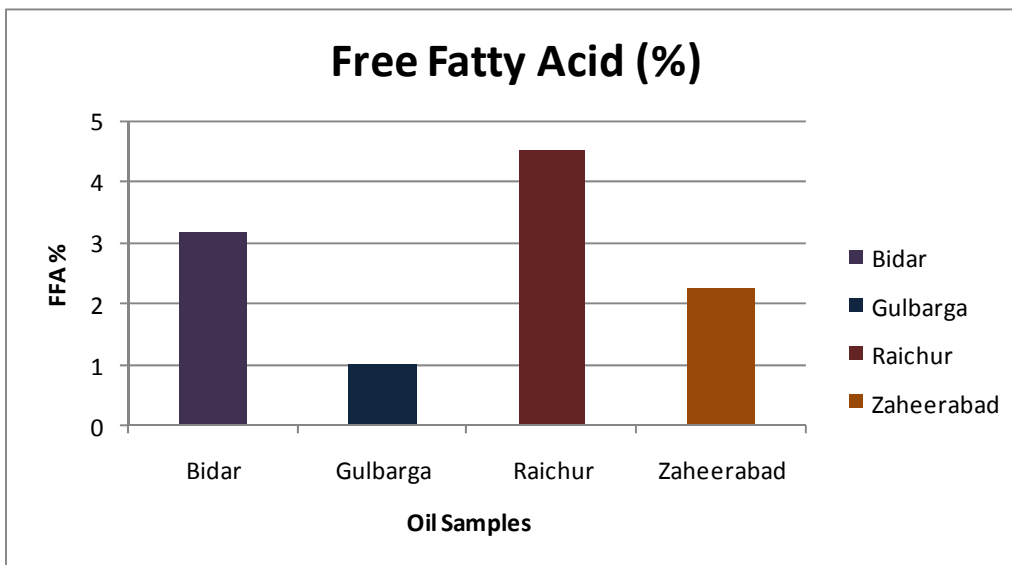


Figure 4: Free fatty acid content of Oil Samples from different area.

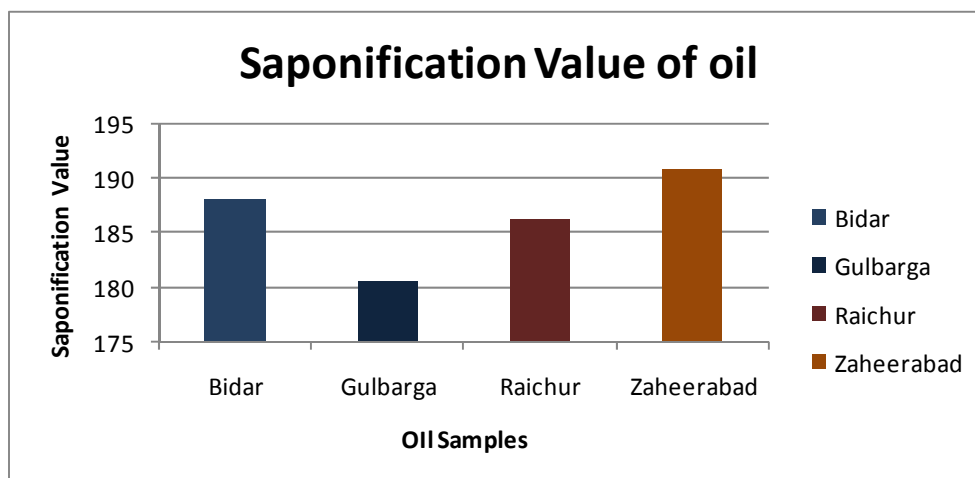


Figure 5: Saponification value of Oil Samples from different area.

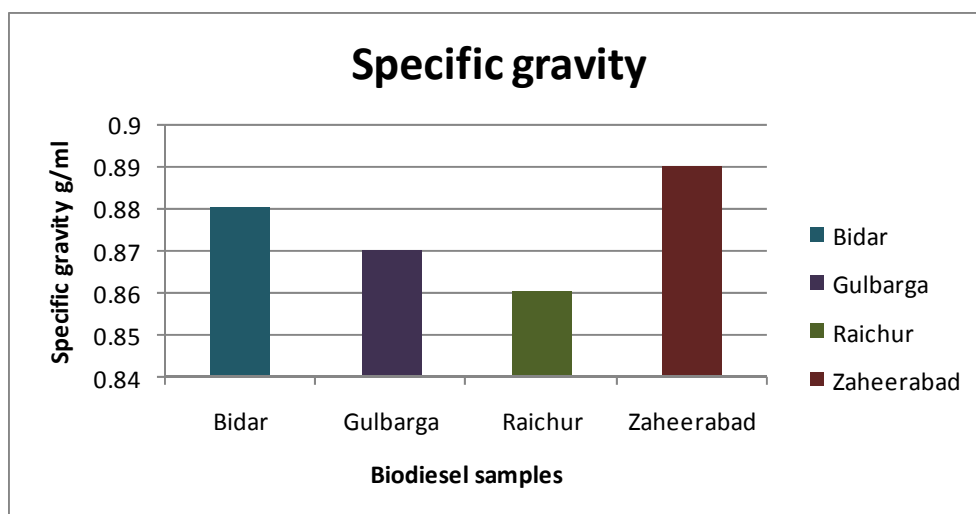


Figure 6: Specific gravity of biodiesel.

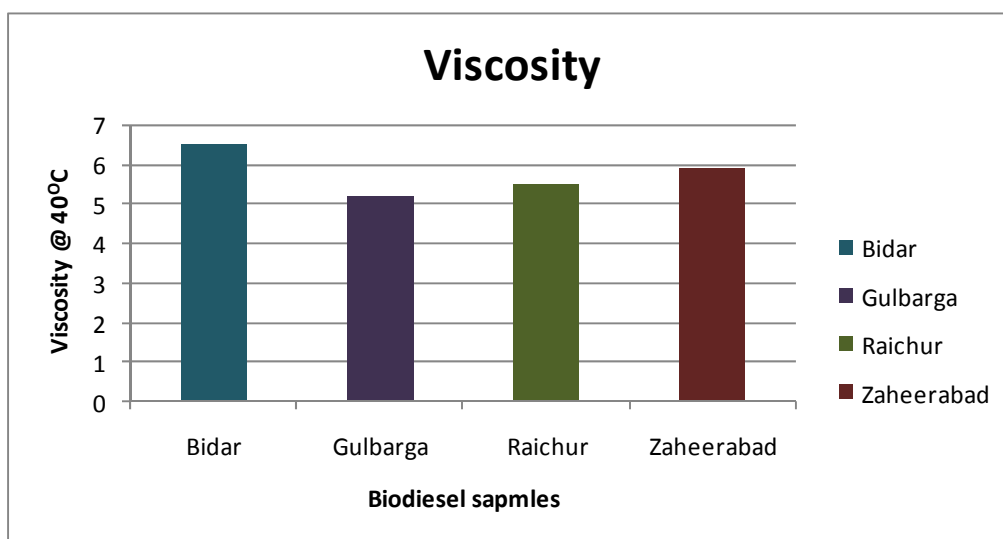


Figure 7: Viscosity of biodiesel.

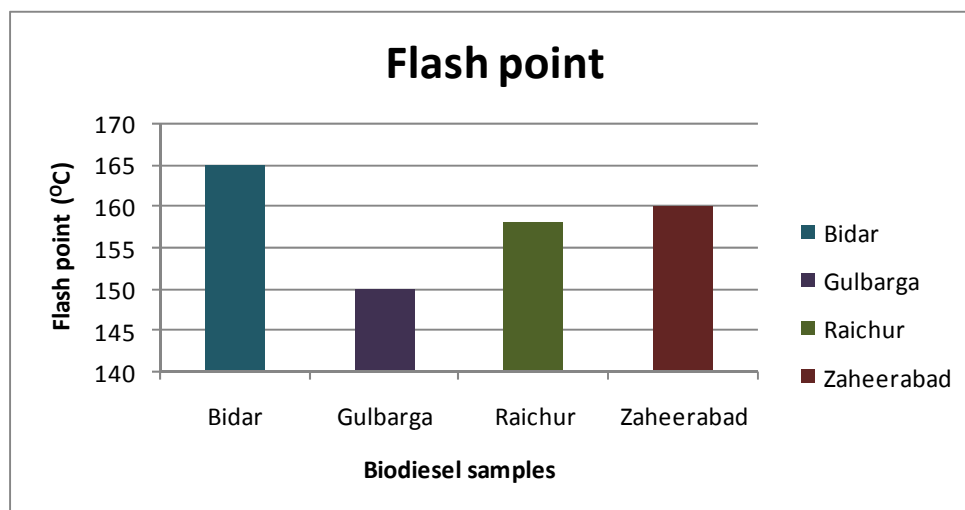


Figure 8: Flash point of biodiesel.

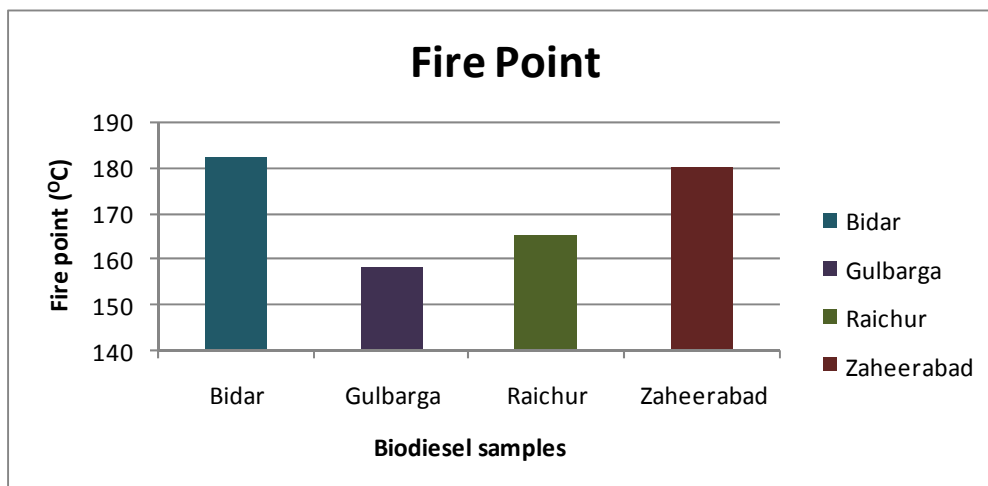


Figure 9: Fire point of biodiesel.

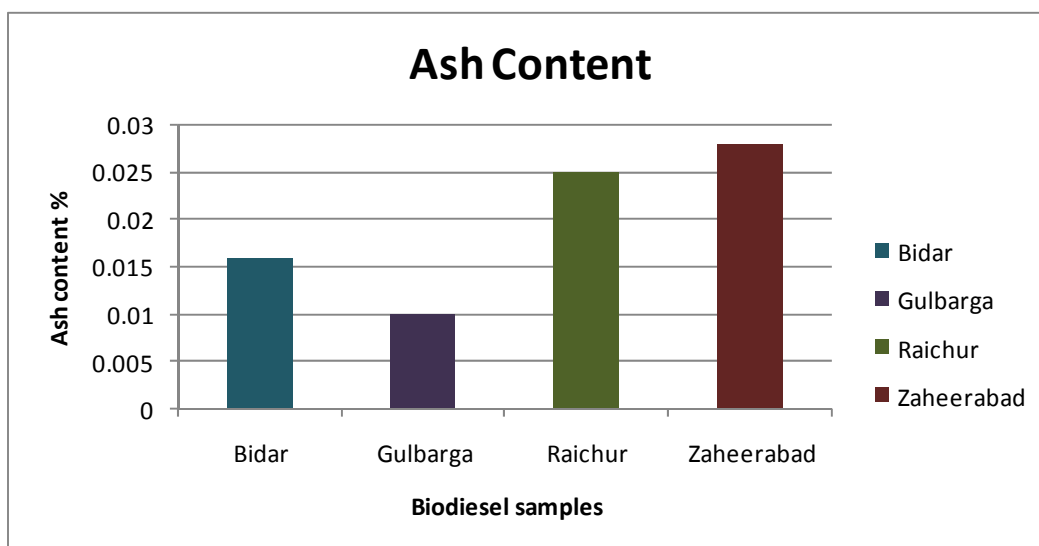


Figure 10: Ash content of biodiesel.

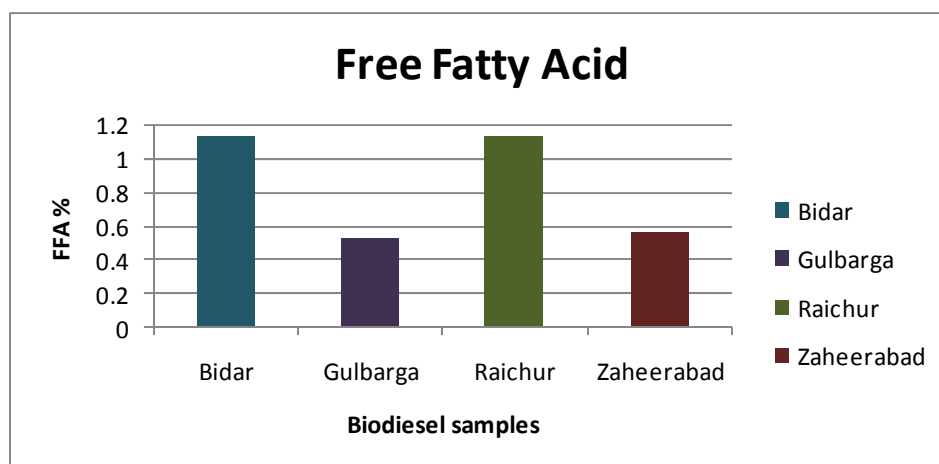


Figure 11: Free Fatty Acid content of biodiesel.

Table 3: Effect of different concentration of NAA and BAP on regeneration of Neem

2,4-D (mg/L)	NAA (mg/L)	KIN (mg/L)	% of Callus initiation
0.1	0.1	0.1	60
0.2	0.2	0.2	72
0.3	0.3	0.3	80
0.4	0.4	0.4	78
0.5	0.5	0.5	50
	BAP (mg/L)	NAA (mg/L)	% of Shoot initiation
	0.1	0.1	--
	0.2	0.2	60
	0.3	0.3	80
	0.4	0.4	55
	0.5	0.5	45

DISCUSSION

The physical properties such as 10-seed weight, oil content and biodiesel content of seeds collected from 30 different biotypes from four districts were studied (Table 1). The maximum 10-seed weight was found in seeds collected from Zaheerabad (7.3 gm). The oil content varied between 25 and 45%. The maximum oil content was observed in seeds from Gulbarga (43%) and minimum was found in seeds from Bidar (31.5 %) (Table 1). Higher oil content of neem indicates its suitability as a non-edible vegetable oil feedstock in oleochemical industries (biodiesel, fatty acids, soap, fatty nitrogenous derivatives, surfactants and detergents, etc.). Soil conditions play a significant role in causing variations in oil yield (Srivastava 1999). Again variation in oil yield may be due to the differences in biotype of plant, cultivation climate, ripening stage and the harvesting time of the seeds (Nzikou et al. 2009). To make biodiesel from neem oil, the base-catalysed transesterification was selected as the process and methyl esters were obtained in the range of 60-85%. Gulbarga seeds were found to have maximum biodiesel content (85%) and minimum was obtained in seeds from Bidar (60%) (Table 1). Density of oil ranged from 0.86 to 0.96 g/cm³. Oil extracted from seeds of Bidar was found to have highest density (0.92 g/cm³), whereas that from Raichur had lowest density (0.89 g/cm³) (Table 2). The density of neem oil was found to be higher than diesel. Higher density means more mass of fuel per unit volume for vegetable compared to diesel oil. The higher mass of fuel would give higher energy available for work output per unit volume. Viscosity of oil obtained was in the range of 41.20-50.32 mm²/s at 30°C. It was found to be maximum in Bidar seeds (50.32 mm²/s) and minimum in Raichur seeds (41.20 mm²/s) (Table 2). Higher viscosity of biodiesel as fossil diesel implies that biodiesel has a lubricating effect in engines which will be an added advantage to the users, since it will reduce wear and tear in the engine. FFA, the major factor for determination of oil quality for biodiesel formation, was found to be maximum in case of Raichur seeds (4.50 %) and minimum in Gulbarga seeds (1.015%) (Table 2). The high FFA content (>2% w/w) favors soap formation and the separation of products will be exceedingly difficult and, as a result, it has low yield of the biodiesel product (Crabbe et al. 2001).

The saponification value of oil extracted was determined and found to be in the range of 180–191g. Maximum saponification value was found in Zaheerabad seeds (190.84 g) and minimum in Gulbarga seeds (180.54 g) (Table 2). The high saponification value reported in this study showed that the oil is normal triglyceride and it is very useful in the production of soap and shampoo (Akbar et al. 2009). High iodine value (81.28 mg/g in Bidar seeds) shows high unsaturation of the oil. The limitation of unsaturation of fatty acid is vital due to the fact that heating highly unsaturated fatty acids results in polymerisation of glycerides which could lead to the formation of deposits.

The flash point and fire point of neem biodiesel were in the range of 125–220°C and 155–260°C, respectively. It was found that oil from seeds from Bidar had maximum flash (165°C) and fire points (182°C). They were observed to be minimum in oil from Gulbarga seeds (flash point 150°C and fire point 158°C) (Table 2). The ash content of neem biodiesel ranged from 0.01 to 0.028%. Zaheerabad biodiesel was found to have maximum ash content (0.028%) and Gulbarga biodiesel had minimum (0.01) (Table 2).

Micropropagation study

Different combinations of NAA, Kinetin and 2,4-D were used for callus initiation (Table 3). NAA (0.3 mg/L), Kin (0.3 mg/L) and 2,4-D (0.3 mg/L) were found to be optimum for obtaining high percentage of calluses. Adventitious shoots were induced on nodal explant in different phytohormone combinations (NAA: 0.1–0.5 mg/L and BAP 0.1–0.5 mg/L). 0.3 mg/L NAA along with 0.3 mg/L BAP was found to be optimum for adventitious shoots regeneration initiation, whereas MS medium with IAA (0.05 mg/L) and IBA (0.05 mg/L) exhibits 80% rooting.

The response shown by different explants varied widely depending on the concentration of NAA and Kin. After two to three weeks of culture of shoots on rooting medium, the small plants were transferred to pots for hardening and acclimatization. Plants acclimatized with a survival rate of 60%.

CONCLUSION

On the basis of the results obtained from all the experiments it was concluded that seeds from Gulbarga district were of better quality with respect to level of oil content, FFA content, and viscosity. The comparison of characteristic fuel properties of biodiesel of neem with the BIS standard and diesel indicates that the produced fuels are comparable with diesel. Neem esters have been found superior in terms of higher flash and fire points. The seed sources in most of the cases were significantly different in oil yield and quality parameters, showing a considerable amount of variability within the distribution range and indicating a good scope of genetic gain through selection. The present study also confirmed that considerable genetic variability exists in neem with respect to oil content and oil quality parameters, which offers scope to the breeder for selection and breeding. Our regeneration protocol could be useful in stabilizing the nursery industry to raise 'clean stock', i.e. rapid clonal propagation of virus-free plants, but the shoot induction frequency is still low for application. Further research is essential to enhance the knowledge base for genetic improvement in neem for quality oil production which could be used in biodiesel production.

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