# Mutation Breeding Strategies for High Energy Tree Species *Prosopis juliflora* in Pakistan

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**Abstract**

Pakistan is facing a severe energy crisis that is negatively impacting the whole economy as this deficiency forced local industries to move to neighboring countries. This also affects the local people losing their jobs. Due to the energy crisis, Pakistanis are forced to use tree biomass for their energy uses. This resulted in deforestation and desertification causing climate change. To combat these issues, increased afforestation of marginal and desert lands is important to meet the local demands. *Prosopis juliflora* is the main species of the desert where it is grown for multiple uses. This study explores mutation breeding's potential to develop useful mutants of *Prosopis* species. Prosopis species are characterized by low biomass, poor tree shape, small diameter, and long needles hinder their widespread adoption. Two types of mutagens i.e., physical (gamma radiation) and chemical (Ethyl methanesulfonate) were used for mutagenesis in *P. juliflora* and *P. cineraria* species. Seeds of both species were treated with three levels of mutagens i.e., low (15mM of EMS, 600Gy of gamma rays), moderate (25mM of EMS, 700Gy of gamma rays), and high (35mM of EMS, 800Gy of gamma rays). Seeds were abraded with sandpaper to enhance seed germination. LD50 for *P. juliflora* was 577.875±67.35 for gamma-ray and 18.732±1.80 mM for EMS. Seed germination was significantly affected in both species. However, *P. cineraria* proved very sensitive to both mutagens as evident by a very low seed germination thus was not suitable for mutant screening. Nevertheless, *P. juliflora* seed germination was significantly affected at high doses as compared to medium and low doses. *P. juliflora* seedlings were selected in the field for superior plant height, plant type, stem shape, and diameter as compared to the controlled seedlings. Selected seedlings were further characterized based on calorific value, lignin, cellulose, hemicellulose, moisture, fresh biomass, and dry biomass. Several superior mutants were selected with desirable characteristics for fuelwood, pulpwood, and afforestation. It is concluded that mutation breeding can be used to develop desirable mutants in a short period as compared to conventional breeding. However, future efforts must be directed to refine mutagenic doses for *P. cineraria* to produce a large population for mutant selection and to develop genotypes.

**Keywords:** *Energy Crisis; Pakistan; Deforestation; Climate Change; Prosopis; Calorific Value; Tree Breeding; Genetic Diversity; Mutation Breeding*

## Introduction

Production of energy has become a major issue globally due to a lack of local resources, and low power production, which is causing a major disruption in energy supply. This has been a main challenge as it is negatively impacting the economic, social, and political sectors of Pakistan (Song et al., 2017). Pakistan's energy crisis, caused by increased electricity demand due to population growth, urbanization, and economic expansion, has shrunk the country's GDP (EAW, 2013; Moral-Carcedo and Vicens-Otero, 2005). Therefore, Pakistan is heavily dependent on fossil oil imports, and increasing currency inflation and disruption of supply have further negatively impacted the economic growth of Pakistan. Many industries were forced to move to neighboring countries such as Bangladesh as a result millions lost jobs. Due to power shortages, most industries and households had to rely on biomass for energy generation. Unfortunately, most of the wood biomass is extracted from natural and state plantations illegally or without implementing conservation and management practices. Pakistan is one of the main deforested countries in the world and faces many risks due to climate change. Climate change is increasing the temperature, disrupting water cycles, causing flooding, and prolonged droughts. A recent, flurry of floods in Pakistan is one of the examples of climate-related anomalies in Pakistan. These climate anomalies are having severe and negative impacts on the productivity challenges in major crops threatening food security in Pakistan.

Forests in Pakistan provide vital ecosystem services, including timber, fuel wood, water regulation, and soil protection, among others (Khan and Mehmood, 2003; Mahmood, 2003; Qazi, 1994). However, with only 4.6 million hectares of forest cover and high rates of deforestation, Pakistan is considered one of the most forest-deficient countries globally (Yaqoob, 2018). Deforestation has caused environmental, floods, health, and financial challenges, including food shortages and high climate risks, making Pakistan one of the top 10 nations facing climate risks in the last two decades (Eckstein et al., 2020). Recent attempts to combat deforestation have failed, leading to extreme weather conditions such as floods, droughts, high temperatures, and heavy snowfalls, exacerbating food shortages and other challenges. Generally, energy is mainly produced from hydro, nuclear, solar, and wind power however the output is not enough to cope with the demand.

Pakistan's energy demand has quadrupled between 2000 and 2020, with an expected doubling by 2030, leading to more oil imports and further economic burden (Shoukat, 2013). Due to the energy shortage and high fuel prices, industries seek cheaper energy sources. In Pakistan, wood biomass is mainly used in the industrial and residential sectors for energy production, and in the pulp and paper industry. Wood energy accounted for 46% of the country's consumption in 1993-94 and typically constitutes nearly 15% of the total primary energy consumption in developing countries (FAO, 1997b; Trossero, 2002). In 2003, Pakistan consumed 43.761 million cubic meters of wood per year, while forest growth was only 14.4 million cubic meters per year, resulting in an annual loss of 29.361 million cubic meters (Pakistan, 2005). Oil may be depleted within 42 years, natural gas in 61 years, and coal in 133 years if the situation persists (Wang and Han, 2011). Pakistan's irrigated and arable lands are mainly used for the production of food crops and fodder. However, there is a vast swathe of land available, that however not suitable for the production of agricultural crops. These lands can be transformed by planting tree species. Therefore, wood from well-managed forests and reforested degraded lands is considered the best alternative to meet the energy crisis.

Drylands cover 41% of the Earth's land surface area (Prăvălie, 2016), including 80% of Pakistan's land area (Shah et al., 2011). Arid soils have low organic matter, high calcium carbonate content, low nitrogen content, weak profiles, and overutilization resulting in desertification (FAO, 2016; Lal, 2009; Safriel et al., 2006). Prosopis species originating from the hyper-arid areas of the world have the potential to survive under these conditions. Moreover, they are most suitable for energy consumption due to their high calorific value. They are also suitable for afforestation due to their high tolerance for drought, waterlogging, and salinity, with enhanced nitrogen to fix nitrogen.

Prosopis is a thorny flowering plant genus in the Fabaceae family, with 45 species distributed across Africa, the Americas, and Asia, including South Asia and Western Asia (Burnham and Johnson, 2004). Four Prosopis species were introduced in Pakistan, including *P. juliflora*, *P. cineraria*, *P. glandulosa*, and *P. farcta*. *P. cineraria* and *P. juliflora* are more widespread in Sindh and Punjab provinces (Essa et al., 2017). *Prosopis juliflora* controls soil erosion, stabilizes dunes, reduces soil salinity, helps in the improvement of soil fertility, and provides fuelwood, construction timber, feed, and food to livestock (Dave and Bhandari 2013; Walter and Armstrong 2014). It is also an excellent source of produce high-quality charcoal (Oduor and Githiomi, 2013). *Prosopis cineraria* is also used as a cattle feed. (Afifi et al., 2018; Yousif, 2012). The pods, bark, flowers, and leaves are used in traditional medicine for the treatment of a variety of health conditions (Pareek et al., 2015). It can also be intercropped with major crops in an agroforestry system (Mahony, 1994).

*Prosopis juliflora* and *P. cineraria* are notorious for having long thorns, multiple stems, crooked stems, and low biomass making it a less preferred species for planting by the farmers. It is therefore important that genotypes with desirable characteristics should be developed. Conventional tree breeding is time-consuming and unsuitable due to long rotational ages, small flower sizes, and polyploidy. On the contrary, mutation breeding, using mutagens such as gamma rays and chemical mutagens such as ethyl methane sulfonate (EMS), offers a good alternative to develop useful mutants (Goel and Behl, 2005; Miller et al., 1984). Several agents that cause mutations are used to induce desirable genetic alterations at high frequencies, including radiation, chemical mutagens, and ionizing agents (Ahloowalia and Maluszynski, 2001). Physical mutagens such as gamma rays have been extremely effective in causing chromosomal aberrations by single or double DNA-strand breaks, or base substitutions. Whereas chemical mutagens like ethyl methane sulfate (EMS), ethyl nitrosourea, dimethyl nitrosamine (DMN), and diethyl sulfate (DES), act basically on nitrogenous base pairs causing different mutations. Over the past 70 years, 2,543 varieties from 175 different plant species, have been developed through mutation breeding worldwide (Chopra, 2005; Maluszynski et al., 2000). Several studies have reported that chemical mutagens are more efficacious than physical mutagens (Rao and Rao, 1983) other experts observed the contrasting behavior (Tarar and Dnyansagar, 1980). However, there are very few examples of mutation breeding in tree species therefore both types of mutagens were employed in this study to examine their efficiency on Prosopis species. The main objectives of the study were to determine the sensitivity of Prosopis species to gamma rays and ethyl methane sulfonate mutagens. Secondly, the selection of genotypes with desirable phenotypic traits such as straight stem form, plant height, high biomass, lignin contents, high calorific value, and thornless genotypes.

## Methodology

To start a tree breeding program, a suitable plus tree must be selected based on its height, diameter, straight bowl, canopy size, crown symmetry, and tolerance to pests and diseases. So, plus trees were identified in various regions of Punjab, Pakistan, with *P. juliflora* being more abundant than *P. cineraria* as *P. cineraria* was observed mostly in wildernesses and graveyards. After identifying the desired plus trees, their seed pods were collected, labeled, and checked for pests and diseases. The seeds were extracted, cleaned, abraded to rupture the hard seed coat, and pre-soaked for 2 hours before being treated with gamma radiation and EMS.

## Mutagenic Treatment

Seeds from both species were treated with physical and chemical mutagen. They were treated with three different doses of both mutagens each treatment was comprised of three replications. Cleaned normal 6000 seeds were treated under three doses of gamma rays 600Gy, 700Gy, and 800Gy and control of 0Gy. Five hundred seeds were treated in each replication. Seeds were at a Cobalt$^{60}$ source at the Nuclear Institute for Agriculture & Biology, Faisalabad, Pakistan. The same procedure was adapted for chemical mutagen treatment where a total of 6000 seeds of both species were treated with 15mM, 25mM, and 35mM of EMS and a control with 0 mM concentration. The seeds were pre-soaked in water overnight before being treated with EMS for 8 hours. Treated seeds were kept at room temperature in labeled plastic trays with filter paper above and below the seeds to keep them moist. Water was replaced daily to avoid any fungus development.

## Seed Germination Percentage

The germination percentage for each treatment was recorded after 8 days and then the final recording after 15 days of treatment, as *P. cineraria* seeds started germinating late compared to *P. juliflora*.

$$\text{Seed germination percentage} = \frac{\text{Seeds germinated}}{\text{Total seeds}} \times 100$$

LD50 for both species was estimated by using the LD50 function of the "*HelpersMG*" package (Girondot, 2019) incorporated in the R programming language (R Development Core Team, 2022).

**Nursery Establishment and Field Plantation**

A nursery was established at Govt. Forest Potted Nursery in Gojra, Punjab. Germinated seedlings were moved to polythene bags and protected from damage by weeding and pest control. A polytunnel was constructed to protect plants from frost damage. The field was prepared according to recommendations, and one-year-old plants were transplanted, watered, and treated with weedicide and pesticide.

**Data collection**

**Phenotypic Data Collection**

Plants were thoroughly observed for mortality rate and phenotypic traits i.e., leaf color changes, plant height, spine size, stem color, stem number, and stem straightness. Three types of phenotypic data were collected i.e., plant height, stem diameter, and number of stems per plant after one year. Plant height was measured with a measuring tape from the ground to the main stem of the plant. Plant stem diameter was measured with a vernier caliper at a height of 4 inches from the ground. The number of stems of each plant was also counted.

**Biochemical Analysis**

Those plants that exhibited desirable phenotypic traits were selected for biochemical analysis. Equal-weight wood samples were collected and prepared for biochemical analysis. Seven parameters were determined i.e., cellulose, hemicellulose, lignin, dry matter, moisture content, fiber content, and calorific value. Moisture content was determined by the oven-dry method per BS EN 13183-1 (BS EN 13183-1, 2002). The crude fiber was determined by the method proposed by Bidwell & Walton (1916). The holocellulose (cellulose and hemicellulose) content was determined by the standard method explained by Timell et al. 1959. While lignin content was determined by Klason method (Galiwango, et al., 2018). To determine the calorific value bomb calorimeter method was used following Nelkon and Parker method (1995).

**Macropropagation of Selected Mutants**

Desirable mutants were mass propagated through cuttings by using auxin to stimulate root development (Atif, unpublished data).

## Statistical Analysis

Phenotypic and biochemical variation from the control treatment was compared by using Analysis of Variance (ANOVA) by using "*agricolae*" package incorporated in the R-program (R Development Core Team, 2022), and means of parameters with a significant difference were compared with Duncan's Multiple Range Test (DMRT).

## Results and Discussion

### General observation

Prosopis species were introduced to Pakistan for dune stabilization and income generation for the poor farmers having marginal lands. *P. juliflora* and *P. cineraria* are the most common Prosopis species, with *P. juliflora* commonly found on roadsides, wastelands, graveyards, and government rangelands. Its sweet pods are eaten by animals, and they help in seed dispersal and germination. *P. cineraria* has small thorns and ash-grey bark and is more common in Southern Punjab. One unique feature was observed in *P. juliflora* that it typically has 3-8 stems per plant, but in some cases, it has a single stem. The seed collection for *P. cineraria* and *P. juliflora* was a challenging task because *P. cineraria* showed a scattered low population while *P. juliflora*. trees possess long thorns making it difficult to collect seeds. Vigorous, disease, and pest-free, and preferably single-stemmed trees with good canopies were selected for seed collection. *P. juliflora* was easy to locate, but finding a plus tree was difficult because of its crooked and multi-stem type. *P. cineraria* trees were hard to find, but those found were usually single-stemmed and vigorous.

### Germination Percentage

Table 1 shows the germination percentage of *P. juliflora* and *P. cineraria* respectively. Gamma radiation doses decreased germination percentage as compared to the control in both species, with the most significant reduction observed at 800Gy. Similarly, high chemical mutagen treatment had a negative effect on seed germination as compared to the control treatment, and the lowest germination was observed at 35mM. The results suggested that high levels of mutagens had a negative impact on seed germination regardless of the species.

**Table. 1.** Comparison of germination percentages of *P. juliflora* and *P. cineraria*

| Treatment | Germination Percentage % | |
|---|---|---|
| | ***P. juliflora*** | ***P. cineraria*** |
| **Physical Mutagen** | | |
| Control | 83.00% | 34.70% |
| 600Gy | 66.00% | 31.02% |
| 700Gy | 52.00% | 19.50% |
| 800Gy | 5.73% | 8.80% |
| **Chemical Mutagen** | | |
| 15mM | 57.00% | 39.10% |
| 25mM | 39.00% | 18.00% |
| 35mM | 19.00% | 16.40% |

**Median Lethal Dose (LD50)**

LD50 was determined in both species based on the relationship between gamma-ray doses and germination percentage. For *P. juliflora* seeds treated under gamma irradiation, the LD50 based on seed viability was 577.875±67.353 (Fig. 1) and LD50 for EMS treatment was calculated to be 18.732±1.803 mM (Fig. 2).

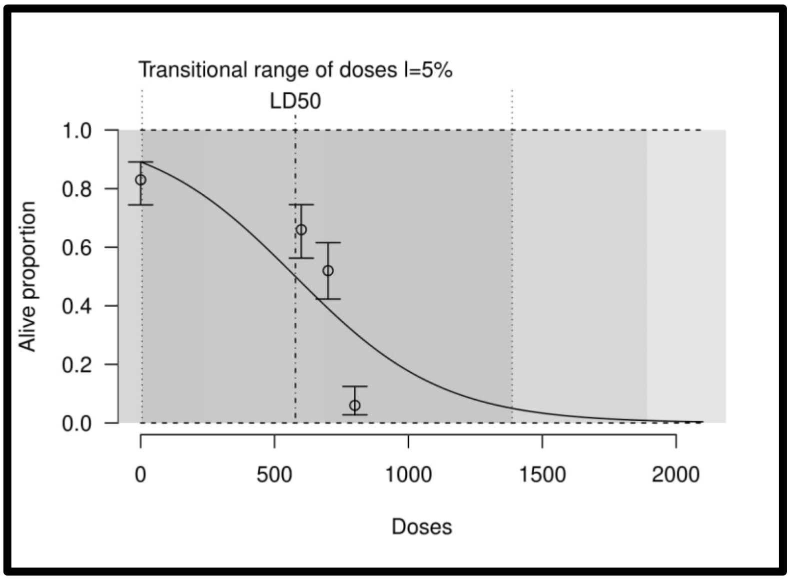


**Figure 1.** LD50 of *P. juliflora* seeds from gamma irradiation treatment

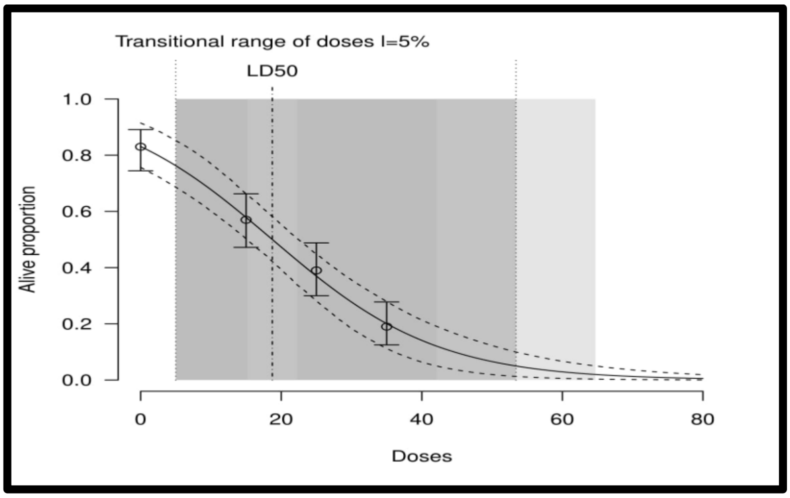


**Figure 2.** LD50 of *P. juliflora* seeds treated with ethyl methane sulfonate (EMS) treatment.

## Plant Mortality

Higher doses of mutagens resulted in higher mortality rates, with the highest at 62.79% in 800Gy gamma radiation. EMS doses showed an upward trend in mortality rate, with the highest at 57.54% from 35mM. *P. cineraria* had the highest mortality rate with all treatments showing above 90% mortality. Plants showed stunted growth, thin stems, and wilting. Among the observed plants, the control group exhibited the least impact, displaying a mortality rate of 90.98%. Conversely, the plants subjected to 800Gy gamma irradiation demonstrated the highest level of susceptibility, resulting in a mortality rate of 97.08%. Due to poor seed germination and a high mortality rate in *P. cineraria*, further studies were carried out on *P. juliflora*.

**Phenotypic Data**

Table 2 displays the findings derived from an investigation conducted to analyze the growth patterns of *P. juliflora* when subjected to various treatment conditions. The mutagenic treatment had a significant effect (F-Value = 380.8, $p < 0.001$) on plant height. The control group had the tallest plants (48.28 cm), while the 25 and 35 groups had the shortest plants 24.91 and 24.95 cm, respectively. The number of stems did not show a significant difference between treatments. For diameter, the treatment also had a significant effect (F-Value = 24.52, $p < 0.001$). The control group had the largest diameter (0.615 cm), while the 800Gy and 600Gy groups had the smallest diameter 0.521 and 0.537 cm, respectively. The 15mM and 35mM had intermediate diameters (0.584, 0.575, and 0.545 cm, respectively). Although the 700Gy treatment group was not the highest significant in terms of plant height, a plant from this group showed the highest stem height of 71cm among all groups including the control. Similar plants also possessed the highest diameter value of 1.2cm (Fig. 3).

Overall, the results suggest that high gamma radiation and EMS doses had a negative effect both on height and diameter. However, some plants from different mutagens treatment groups showed superior growth in height and stem diameter. The number of stems per plant was not significantly affected by either treatment or concentration. The control group showed the best growth performance in terms of plant height and diameter.

**Table. 2.** Comparison of phenotypic parameters of *P. juliflora* through Analysis of Variance (ANOVA).

| Parameter | SOV | Df | SS | MS | F Value | Duncan's Mean Comparison Test | |
|---|---|---|---|---|---|---|---|
| Plant Height | Treatment | 6 | 139093 | 23182 | 380.8*** | Control | 48.28a |
| | | | | | | 600Gy | 34.05b |
| | | | | | | 15mM | 31.01c |
| | | | | | | 700Gy | 29.40c |
| | Residuals | 3221 | 196086 | 61 | | 800Gy | 26.40d |
| | | | | | | 35mM | 24.95e |
| | | | | | | 25mM | 24.91e |
| Number of Stems | Treatment | 6 | 2 | 0.03074 | 0.153 ns | | |
| | Residuals | 3221 | 6474 | 2.0099 | | | |
| Diameter | Treatment | 6 | 2.41 | 0.4021 | 24.52*** | Control | 0.615a |
| | | | | | | 700Gy | 0.584b |
| | | | | | | 15mM | 0.575b |
| | | | | | | 35mM | 0.545c |
| | Residuals | 3221 | 52.81 | 0.0164 | | 25mM | 0.538c |
| | | | | | | 600Gy | 0.537c |
| | | | | | | 800Gy | 0.521c |

* p≤0.05 ** p≤0.01 *** p≤0.001


**Figure. 3.** Growth comparison of A) *P. juliflora* plant treated with 700Gy of gamma radiation B) Control *P. juliflora* plant.

**Gamma Irradiation and Biochemical Parameters**

This table (Table 3) presents the results of an analysis of variance (ANOVA) for biochemical parameters of *P. juliflora* plants treated under gamma radiation. From the table, we can see that there are significant differences between the treatment groups for some of the parameters. For example, the dry matter content and moisture content show significant differences between the treatment groups ($p < 0.05$) and control, while the crude fiber, cellulose content, and hemicellulose content do not show significant differences ($p > 0.05$). The lignin content and calorific value also show significant differences between the treatment groups and control ($p < 0.05$).

**Table 3.** Comparison of biochemical parameters of *P. juliflora* plants treated under gamma radiation through Analysis of Variance (ANOVA).

| Parameter | SOV | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| Dry matter content | Treatment | 3 | 3.234 | 1.0782 | 5.433 | 0.00905** |
| | Residuals | 16 | 3.175 | 0.1984 | | |
| Moisture content | Treatment | 3 | 3.234 | 1.0782 | 5.433 | 0.00905** |
| | Residuals | 16 | 3.175 | 0.1984 | | |
| Crude Fiber | Treatment | 3 | 1.88 | 0.6267 | 0.241 | $0.867^{ns}$ |
| | Residuals | 16 | 41.62 | 2.6013 | | |
| Cellulose Content | Treatment | 3 | 8.87 | 2.956 | 0.504 | $0.685^{ns}$ |
| | Residuals | 16 | 93.83 | 5.865 | | |
| Hemicellulose Content | Treatment | 3 | 12.25 | 4.084 | 0.587 | $0.632^{ns}$ |
| | Residuals | 16 | 111.29 | 6.955 | | |
| Lignin Content | Treatment | 3 | 26.19 | 8.73 | 3.444 | 0.042* |
| | Residuals | 16 | 40.56 | 2.535 | | |
| Calorific Value | Treatment | 3 | 26820 | 8940 | 3.374 | 0.0446* |
| | Residuals | 16 | 42400 | 2650 | | |

**EMS Treatment and Biochemical Parameters**

Table 4 showcases the outcomes obtained from an analysis of variance (ANOVA) performed to assess the biochemical parameters of *P. juliflora* plants subjected to EMS treatment. Based on the data presented in the table, it is evident that no statistically significant differences exist among the treatment groups for any of the parameters examined ($p > 0.05$). The dry matter content, moisture content, crude fiber, cellulose content, hemicellulose content, lignin content, and calorific value do not show significant differences between the treatment groups. Therefore, the EMS treatment does not seem to have a significant effect on the biochemical parameters of *P. juliflora* plants.

**Table 4.** Comparison of biochemical parameters of *P. juliflora* plants treated with EMS through Analysis of Variance (ANOVA).

| Parameter | SOV | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| Dry matter content | Treatment | 3 | 1.23 | 0.41 | 1.829 | 0.174$^{ns}$ |
| | Residuals | 20 | 4.483 | 0.2242 | | |
| Moisture content | Treatment | 3 | 1.23 | 0.41 | 1.829 | 0.174$^{ns}$ |
| | Residuals | 20 | 4.483 | 0.2242 | | |
| Crude Fiber | Treatment | 3 | 12.04 | 4.014 | 1.469 | 0.253$^{ns}$ |
| | Residuals | 20 | 54.64 | 2.732 | | |
| Cellulose Content | Treatment | 3 | 10.86 | 3.621 | 0.659 | 0.587$^{ns}$ |
| | Residuals | 20 | 109.94 | 5.497 | | |
| Hemicellulose Content | Treatment | 3 | 28.46 | 9.486 | 1.197 | 0.336$^{ns}$ |
| | Residuals | 20 | 158.54 | 7.927 | | |
| Lignin Content | Treatment | 3 | 14.83 | 4.944 | 1.825 | 0.175$^{ns}$ |
| | Residuals | 20 | 54.18 | 2.709 | | |
| Calorific Value | Treatment | 3 | 6553 | 2184 | 0.873 | 0.472$^{ns}$ |
| | Residuals | 20 | 50071 | 2504 | | |

**Selection of Superior Genotypes**

Table 5 shows selected mutant plants of *P. juliflora* with different phenotypic and biochemical parameters. Several useful mutants were selected exhibiting desirable traits such as plant height, diameter, and low and high lignin contents. Plant a379, from 600Gy gamma rays' treatment, shows the highest number of stems per plant, which is 8. A mutant b127, from 700Gy treatment, showed a single stem and the highest lignin and calorific value. Another plant b253 from the same group resulted in the highest value of dry matter and cellulose. Mutant y203, from 25mM EMS treatment, showed 0.4cm diameter but the same plant gave 5000kcal/kg calorific value and 6 stems. A mutant from 35mM EMS treatment showed 7 stems-per-plant, it also possessed the lowest lignin content of 12.6% and the lowest calorific value was 4790kcal/kg, making it a suitable choice for the paper industry.

**Table 5.** Phenotypic and Biochemical data of selected mutant *P. juliflora* plants

| | | **Phenotypic Data** | | | **Biochemical Data** | | | | | | **Calorific Value** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Tag #** | **Treatment** | **Height (cm)** | **No. of Stems** | **Diameter (cm)** | **% Dry matter** | **% Moisture content** | **% Crude Fiber** | **% Cellulose** | **% Hemicellulose** | **% Lignin** | **Calorific Value (Kcal/kg)** |
| a379 | 600Gy | 46 | 8 | 0.7 | 95.5 | 4.5 | 66 | 46 | 23.2 | 14 | 4860 |
| b127 | 700Gy | 71 | 1 | 1.2 | 97 | 3 | 65.3 | 41.7 | 29 | 19.3 | 5040 |
| b253 | 700Gy | 25 | 7 | 0.5 | 97 | 3 | 64.9 | 47 | 24.3 | 18.5 | 5000 |
| y203 | 25mM | 31 | 6 | 0.4 | 95.8 | 4.2 | 66 | 38.9 | 25.9 | 18.8 | 5000 |
| z75 | 35mM | 29 | 7 | 0.4 | 96.6 | 3.4 | 66.5 | 43.5 | 30.5 | 12.6 | 4790 |

## Discussion

Prosopis was introduced in Sindh, Pakistan for afforestation but became an invasive weed due to its deep roots and drought tolerance. It is still preferred for afforestation in low-water availability and high-temperature regions. Its uses include fuelwood, charcoal, and fodder. Its drawbacks are its short height, low biomass, stem diameter, invading nature, long thorns, and multi-stem characteristics. So, after an in-depth and detailed observation of its uses among local communities and afforestation potential, the main purpose of conducting this study was the selection of mutants with desirable features such as a single stem, large diameter, high and low calorific value, and lignin contents, and thorn lessness. The evaluation of the LD50 of a substance or mutagen is necessary to identify any detrimental effects resulting from deliberate or accidental short-term exposure (Clemedson et al., 2000). So, LD50% for both mutagens was determined. LD50% of gamma rays for Prosopis were 750Gy as determined by Asif et al. (2020) and LD50% of EMS was studied in this experiment, which was 25mM.

Physical and chemical mutagens negatively affected seed germination due to their destructive features. Low gamma irradiation dosages resulted in high seed germination percentages, but this decreased at higher doses (700Gy and 800Gy). *P. cineraria* had the lowest germination percentage even among the control treatments. These results are like the findings of many previous investigators who described that higher doses of gamma radiation decrease seed germination percentage in different crop and tree plants. For example, a very high dose of 50Gy of gamma rays on barley seeds by Wang et al. (2017) suppressed seed germination percentage. Similarly, a 400Gy dose in fenugreek by Hanafay and Akladious (2018), a 200Gy dose in *Zea mays* by Marcus et al. (2013), and a 300Gy dose of gamma rays by Norfadzrin et al. (2007) in tomato seeds resulted in lower germination percentage as compared to lower doses. Goel and Behl (2005) also documented a corresponding inverse relationship in the seed germination percentage and gamma radiation dose in seeds of two semi-arid tree species, *Prosopis juliflora*, and *Acacia nilotica*.

EMS treatment showed the same trend that higher doses produced low germination. A similar result was obtained by Laskar et al. (2018) when they treated tomato seeds with EMS and recorded low germination in M1 at higher concentrations. Some more examples of studies with corresponding results of low germination from higher EMS concentrations are Ambli and Mullainathan (2014) in pearl millet, Anbarasan et al. (2013) in sesame, Ariramana et al. (2014) in pigeon pea, Gnanamurthy et al. (2011) in maize, Satpute and Fultambkar (2012) in soybean, Talebi et al. (2012) in rice and Talebi et al. (2012) in cluster bean. This decrease in germination against higher doses of gamma rays can be due to subsequent mitotic abnormalities, seed dormancy, moisture content, seed maturity, seed tissue damage, and chromosome retardation (Al-Safadi and Simon 1990; Datta, 2009). Similarly, Khan and Goyal (2009) stated that this low germination can be due to altered enzyme activity or damage to cell components.

Plant mortality increased with increasing doses of EMS and gamma radiation in *P. juliflora*. Control group mortality was far lower 800Gy of gamma radiation and 35Mm of EMS. *P. cineraria* had high mortality rates (above 90% in all mutagen treatments). Mortality started at 800Gy gamma irradiation and 35mM EMS, moving down towards lower doses, with control plants being the last to die. These results are in accordance with many studies carried out previously as a treatment of high doses in Withania by Das et al. (2010) decreased the plant survival rate. Soybeans showed a high mortality rate in M1 and M2 after treatment with gamma radiation and mortality increased with dose (Mehetre and Kshirsagar, 2022). Goel and Behl (2005) also documented the direct relation of increasing doses with mortality rate. Similarly, cowpeas by Girija and Dhanavel (2009) and horse gram by Kulkarni (2011), chickpeas by Barshile (2006) were treated with different EMS concentrations and all these researchers recorded low plant survival at higher doses of EMS. This reduction in plant survival can be due to severe damage to the plant cells, as reported by Sikora et al. (2011) that point mutation at random sites is caused by EMS which retards many processes of protein synthesis.

Gamma rays and EMS concentration affected the stem heights of *P. juliflora* plants. Lower doses of both mutagens resulted in greater mean stem heights than higher doses. Physical mutagen at a low dose of 600Gy resulted in more mean height than a low dose of 15mM EMS. In general, higher doses resulted in lower stem height. These findings are in accordance with previous studies. Gamma radiation treatment by Irfaq and Nawab (2001) on three wheat varieties resulted in a decrease in the survival rate and a delay in germination. Plant height also decreased with a higher dose of gamma rays. Similarly, Kalia et al. (2001) in bread wheat, Gonzales (2007) in orchids, and Yaping, (1996) in chickpeas reported the same behavior of plant height reduction at higher doses of EMS. Shah et al. (2008) stated that some processes like changes in ascorbic acid content, biochemical and physiological disturbances, and destruction of auxin, can cause inhibition of plant development and germination rate. In consensus with our findings on plant height, Behera et al. (2012) also reported some morphological effects of EMS treatment like leaf size and plant height in *Asteracantha longifolia*. Many previous studies suggested that low plant height at higher dosages of mutagens can be due to the reason of abnormal production of different signaling hormones like indole acetic acid, gibberellin, or strigolactones (Bennet et al*.,* 2014; Bishop, 2003, Kwon and Choe, 2005; Liu et al., 2010 and Sazuka et al., 2009).

Physical mutagen significantly affected the *P. juliflora* plant stem diameter positively and the diameter increased from lower to higher doses of gamma radiation. On the other hand, EMS or chemical mutagen showed a negative or declining trend in mean stem diameters. Higher doses of EMS produced lower stem diameters as the lowest dose of 15mM gave the highest mean stem diameter. Similar results were also obtained by Wi et al. (2007) as larger stem diameters were noticed from higher doses of 8krad of gamma radiation. Goel and Behl (2005) also observed an increase in diameter at higher doses of gamma rays. Some other researchers like Fu et al. (1999) stated the opposite results as diameter decreased for higher doses of gamma radiation. Chaomei and Yanlin (1993) and Melki and Marouani (2010) also reported a decrease in plant vigor at higher doses. This increase in stem diameter is possibly because gamma rays negatively affected the stem height from lower to higher doses which forced lateral meristematic growth which resultantly increased the stem diameter. Behera et al. (2012) reported the detrimental effects of EMS on plant vigor at higher doses which were like our results of EMS effect on plant diameter. Ibrahim (2008) tested EMS effects on strawberries and reported the same behavior of higher doses (reduction in plant vigor).

The number of stems per plant was not affected negatively or positively, neither by gamma radiation nor by ethyl methanesulphonate, as all the groups showed nearly the same mean value for the number of stems per plant (3.93 to 4.06) in comparison to the control group's value of 4.08. Previous studies by different researchers reported three types of contrary effects (no effect, increasing, and decreasing) on stems per plant by increasing the dose of gamma irradiation. Tatiana et al. (2019) reported no significant variation in stem number in *Calendula officinalis* from different treatments of chemical mutagens. Kaul and Bradu (1972) found an increasing number of stems or branches per plant after irradiation treatment of seeds of *Atropa belladonna*. Similarly, Alba et al. (2004) in *Chamomilla recutita*, and Youssef et al. (2000) in geranium obtained the same results. In the case of chemical mutagenesis, Jabeen and Mirza (2002), and Sri Devi and Mullainathan (2011) reported the same response of an increasing number of branches or stems at higher doses of EMS. In contrast to these findings, Marizka (2010) in soybean, and Shukla and Datta (1993) in Chrysanthemum reported that mutagenic dose and the number of stems per plant are inversely proportional. To put it simply, increasing the gamma radiation dose decreased the number of branches or stems. Stem numbers did not show significant variation, indicating the potential for generating mutants with desirable height and diameter characteristics. Therefore, the same mutagenic dosage can be used to develop mutant *P. juliflora* plants with improved height and diameter without altering stem numbers. Although, there was no significant difference among the mean number of stems of different treatment groups a plant from 600Gy gave 8 stems per plant. This trait could be useful in arid area afforestation due to their wider canopy and greater biomass production.

The dry matter content of *P. juliflora* plants increased gradually from 0Gy to 700Gy, then declined to 800Gy. EMS-treated groups exhibited increased dry matter content mean values for all dosages. However, the difference among chemical mutagen doses was insignificant. These findings are consistent with previous research, which demonstrated that radiation improves dry matter content until a specific dose, after which it declines at higher lethal doses. Sarker et al. (2014) reported a decrease in total dry matter content in soybeans at higher doses of 300Gy, 400Gy, and 500Gy of gamma rays as compared to control and lower doses of 200Gy. Some other investigations by Bamidele and Akanbi (2013), Linda et al. (2020), and Rao et al. (1994) did not report any significant effect on dry matter percentages from different levels of gamma radiation dosages. Mutants with increased dry matter content are valuable in this regard because dry matter content is a crucial indicator of nutrient availability and energy content in wood. It is also essential for biomass production, carbon sequestration, and pulp production. Plants with higher dry matter content are preferred in the paper industry, as the water content is considered waste in pulp production.

Gamma radiation did not significantly affect the moisture content percentage in *P. juliflora* plants. The lowest dose of 600Gy slightly decreased the moisture content as compared to the control group. Then 700Gy gave a lower moisture content as compared to the last dose of 600Gy but the 800Gy group gave the highest mean value of 4.25% (more than the control). In the case of ethyl methanesulfonate, all dosages generated a mean value between 3.53% (35mM) to 4.11% (control) so the overall effect was non-significant for both types of mutagens. These results are in accordance with the findings of Bamidele and Akanbi (2013), Linda et al. (2020), and Rao et al. (1994) as they obtained no significant effect of gamma irradiation on moisture content in *Cajanus cajan*. However, some other researchers Arthur and Wiendl, (2000) and Premi Devi et al. (2018) reported contrary results of the significant reduction in moisture content at higher doses of gamma radiation. The moisture content of wood is very important from the perspective of the paper industry as well as for plantation projects in drier conditions of arid or semi-arid areas. Plants possessing a higher percentage of water content due to their genetic mutations would prove to be advantageous for cultivation in regions experiencing water scarcity. This is because they possess the capability to retain a larger quantity of water during the rainy season. Plants with less moisture content would be a good choice for pulp and paper manufacturing and timber purposes. Because high water content causes timber wood to shrink and then expand.

Crude fiber percentage was not significantly affected by gamma radiation. However, the highest dose of 800Gy slightly decreased the fiber content. So, in general, all the treatments produced almost the same mean fiber percentage. EMS dose of 35mM gave the highest mean fiber value and the lowest dose of 15mM produced the lowest mean value among all the groups. As all mutagenic treatments failed to induce significant differences, the crude fiber remained unaffected by the treatments. Findings by Ihsanullah et al. (2005) and Mohammadzai et al. (2010) were in support of our results as they observed no significant change in crude fiber content in dates, both with lower and higher doses of gamma radiation. In contrast to these results, some researchers like Mohammadzai et al. (2010) and Abdelwhab et al. (2009) reported a negative effect on crude fiber percentages at higher doses when they treated beans and palm dates under gamma exposure, respectively. There are no or very few studies on crude fiber percentage from EMS treatments. However, Al-Rawi and Kohel (1970) and Witt et al. (2018b) reported the positive effect of EMS on different traits of fiber i.e., improvement of fiber quality, length of fiber, strength, and elongation of fiber in cotton.

Gamma radiation and ethyl methanesulfonate did not affect the holocellulose content significantly. However high doses of both mutagens reduced the holocellulose content as compared to lower dosages. Some previous studies, Ajila et al. (2007), Emaga et al. (2008), and Orozco et al. (2012)

also showed degradation of holocellulose at higher doses of gamma rays. Similar results of reduced cellulose were obtained by Purente et al. (2020) from different higher concentrations of ethyl methanesulfonate. As in our study cellulose content of *P. juliflora* in both types of mutagenic treatments was higher than the control group at lower doses so 600Gy dose of gamma radiation and 15mM dose of EMS can be further used to develop a mutant with higher cellulose content which can be used for pulp production or paper making. According to Molin and Teder (2002), a high ratio of cellulose to hemicellulose increases folding endurance and fracture toughness. Hemicellulose on the other hand can be used for sorbitol (pharmaceutical sweetener) production by reduction process and alcohol production by fermentation (Gírio et al*.,* 2010).

Lignin percentage was affected significantly by gamma radiation and a moderate dose of 700Gy gave the highest mean value of 17.88%. A non-significant effect was observed by EMS treatment, however, the highest dose of 35mM decreased the lignin content as compared to the control and other groups. Results from physical mutagenesis are corroborated by the findings of Chin et al. (2017) where lignin content decreased at higher doses of gamma radiation. Similarly, the results of Purente et al. (2020) support our findings that lignin decreased by increasing EMS dosages. In the paper industry, the removal of lignin from pulp is not specifically targeted for application purposes but to enhance the paper quality (Cotana et al., 2014) because lignin gives yellowish color to paper. So, lignin is removed from wood pulp by Kraft pulping, soda pulping, or sulfite pulping methods (Haq et al., 2020). But these methods are harsh, expensive, and time-consuming so mutants with low lignin content can be used for pulp production. On the other hand, lignin is positively and significantly correlated with calorific value. This mutant with high lignin content can be used as fuelwood as reported by Beauchet et al. (2012). Lignin can be yielded (from pulp) from mutants with high lignin percentage which can be used as a biofuel, bioplastic, biofertilizer, value-added chemicals, food additives, animal feed, textile, personal care product, adhesives, and lubricants (Cho et al., 2018; de Wild et al., 2017). Moreover, improved genotypes with high biomass yield and better phenotypic traits would be accepted and preferred by the local community.

Changes in calorific values in different wood species can be accredited to the biochemical composition of the wood. Some extractive contents and most importantly lignin percentage show a notable influence on calorific value. As stated by Kaltschmitt and Streicher (2009), the net heat of combustion from lignin is 27.0 MJ/kg while cellulose and hemicelluloses have 17.3 and 16.2 MJ/kg net heat of combustion, respectively. Similarly, Fuwape (1989) obtained 25.4 and 19.7 MJ/kg gross-heat combustion values for lignin and cellulose in *Gmelina arborea*. Therefore, the higher the lignin content, the higher will be the calorific value (Demirbas, 2001; Kataki and Konwer, 2001). Moreover, Krauss et al. (2011) and Wagenführ (2007) corroborated these results by getting a higher net heat of combustion value of 19.1 MJ/kg from Santos rosewood and a lower value of 16.6 MJ/kg from Birchwood because Santos rosewood has approximately 31.2 % lignin while Birchwood possesses lignin content between 19.3 and 27.4 percent. According to Demirbas (2001), the higher heating value (HHV) of any lignocellulosic fuel from any plant species is in fact a function of its percentage of lignin. Howard (1973) reported that cellulose and hemicelluloses possess a higher heating value of 18.60 kJ/g, on the other hand, lignin alone has about 23.26 to 25.58 kJ/g HHV.

For mutant plant selection, this study used phenotypic traits like height, diameter, and number of stems. Straight and tall single-stemmed plants were selected for timber production, while lower-height, higher-diameter plants were chosen for biomass. Multi-stemmed plants were picked for afforestation and reforestation projects and were found to have more green foliage, suitable as forage in areas with shortages. Selected mutants underwent biochemical analysis for desirable traits. High-lignin plants were good for energy production, and low-lignin for pulp and paper. Plants with high holocellulose levels were ideal for biomass, and high moisture for arid lands. Mutants with high calorific values were suitable for charcoal production or fuel. These traits have the potential for economic and environmental benefits in various sectors.

In this study gamma radiation proved to be more effective as compared to EMS treatment on seeds of both Prosopis species. The following reasons provide insights into the increased effectiveness of gamma radiation in comparison to chemical mutagens. Gamma radiation can cause more extensive DNA damage and a higher incidence of mutations because it has high energy and can enter the target tissue deeply. Chemical mutagens, like EMS, on the other hand, frequently have limited ability to enter the target cells or tissues. For example, a study on tomatoes by Shirasawa et al. (2016) stated that gamma rays caused a higher percentage of deletions and insertions in the tomato genome as compared to EMS.

As mentioned earlier, gamma radiation induces a variety of mutations, including translocations, large deletions, and chromosomal aberrations which result in more unique changes in phenotype or gene expression. On the other hand, chemical mutagens mostly induce point mutations, which result in less effect on gene function. Xiao et al. (2019) stated that EMS usually induces nucleotide modifications causing mispairing of C with T, which leads to the transition of CG to TA. Mutagens can differ in their level of effectiveness for different species. This is because of transformations in repair mechanisms of genetic makeup. For instance, the Sofu cultivar of *Triticum durum* showed a higher frequency of mutation when treated with gamma irradiation as compared to EMS treatment, while another cultivar Gediz-75 from *Triticum durum* displayed a higher mutation rate when treated with EMS (Sakin and Sencar, 2002).

Different cells or tissues of a plant species can have different sensitivities to different mutagenic agents. For instance, some tissues may be more sensitive to chemical mutagens, while some other cells or tissues may be more susceptible to gamma rays. Effectiveness may decrease or increase when a combination of different mutagens is used. A study on tomatoes showcased the highest effectiveness of gamma radiation followed by sole EMS and a combination of EMS and gamma radiation treatments (Sikder et al., 2013). Multiple copies of each chromosome exist in polyploid plants, which can enhance the risk of genetic alterations brought on by mutations and provide genetic redundancy. As a physical mutagen, gamma radiation can alter chromosomes in a variety of ways, including deletion, translocation, inversion, and duplication, which increases genetic variability in polyploid plants. EMS, in contrast, mostly causes point mutations, which are less likely to result in significant genetic changes in polyploid plants. Quantitative traits are controlled by multiple genes so mutations like point mutations by EMS affecting just one or few genes may not develop a significant alteration in plant phenotype especially in polyploids like *P. juliflora*. Nevertheless, gamma radiation with its broad spectrum of effects and power can cause transformations in multiple genes at the same time, resulting in noteworthy mutations in quantitative characters. While EMS induces just point mutations, resulting in less substantial vagaries in phenotype.

The composition of alleles may offer a different response of plant species to mutagens. While gamma rays induce mutations in dominant as well as recessive alleles, EMS primarily causes mutations in recessive alleles making EMS less effective in this case. Therefore, gamma rays generate a broader range of mutations enhancing the possibility of obtaining beneficial traits. Finally, gene action can also affect the mutagenic response of plant species. Gamma radiation can induce qualitative along with quantitative mutations in gene expression, but EMS mutations mainly affect negligible quantitative alterations in gene expression. Therefore, gamma radiation produces diverse and extensive mutations in gene expression, which may result in increased probabilities of getting advantageous characteristics.

## Conclusions

*P. juliflora* has a narrow genetic base and its importance can be enhanced by using breeding techniques but due to the long rotation age of trees, it is a very difficult and time-consuming task to use conventional breeding techniques. So, mutation breeding and biotechnology techniques are a good way to obtain good mutants with desirable phenotypic characteristics. Mutation breeding has been successfully used in crops to produce new varieties in a short period. Mutation breeding has only recently been used in tree species. Both physical and chemical mutagens are used in a mutation breeding program. However, physical mutagens are commonly used due to ease and safety as compared to chemical mutagens. Gamma rays are preferred as a physical mutagen whereas EMS is commonly used as a chemical mutagen. Both mutagens were used in this study due to their different modes of action on the genome. Physical mutagen proved more effective in inducting genetic diversity as compared to chemical mutagen. This could be due to the mode of action of these mutagens and the hard seed coat of the species. *P. juliflora* is found commonly and widely thriving in nature as compared to *P. cineraria*. A restricted distribution in the wild could be attributed to a very low seed germination rate that was observed in *P. cineraria* as compared to *P. juliflora*. As a result of low seed germination and low population size, further studies were conducted on *P. juliflora*. A wide range of responses was observed when *P. juliflora* seeds were treated with mutagens, such as low seed germination, high mortality, stunted growth, plant height, stem diameter, and difference in biochemical composition of the mutants. Several mutants with desirable traits such as tall and short, different stem diameters, low to high cellulose content, variable lignin content, and calorific value were successfully selected. These mutants prove superior to the control plants. This showed that mutation breeding can be used in tree species to develop desirable genotypes in a short period. Selected mutants were asexually multiplied for field planting and evaluation.

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