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Short Communications

Determination of semi-lethal dose of colchicine on *in vitro* grown callus of *Azadirachta indica* genotypes

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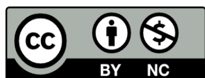

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Abstract: Colchicine is a toxic mutation-inducing chemical substance widely used to induce polyploidy for plant improvement. Being toxic, dose estimation to plant tissue is necessary for polyploidy induction studies. LD50 dose or the semi-lethal dose is the amount of a toxic substance that can kill half of the biological test sample in a single application. It is generally helpful to estimate the toxic nature of a chemical substance. In the present study, the callus of three genotypes FRIH12, FRIH22, and AFRIC1 of *Azadirachta indica* (neem) was incubated on an MS medium with various doses of colchicine under in vitro conditions. The survival percentage of callus of each genotype under varying concentrations of colchicine was observed. Ocular toxicity, mathematical (Spearman-Karber), and statistical (Miller-Tainter) methods were used to determine the LD50 dose of colchicine for the three genotypes. Miller-Tainter method is the most efficient and accurate for determining the LD50 dose of colchicine and for the three genotypes FRIH12, FRIH22, and AFRIC1, the LD50 dose was found to be 50.1mg/l, 60.3mg/l and 50.1mg/l respectively. Amongst genotypes, FRIH22 was most resilient against the treatments of colchicine.

Keywords Neem; genotypes; *Azadirachta indica* A. Juss; *in vitro*; colchicine; LD_{50}; semi-lethal dose

1. Introduction

Azadirachta indica A. Juss. (Neem or Margo) is an important tropical tree, with established therapeutical and insecticidal properties and is indigenous to the Indian sub-continent [1, 2]. It is called the miracle tree due to its manifold benefits in the field of agriculture [3, 4]. Neem oil squeezed from the seeds and fruits of this tree, is known as a powerful biopesticide and is very effective in organic cultivation [5]. Oil and neem seed cake, granules, leaves, and bark are used as fertilizer, soil conditioner, fumigant, pesticide, and lately as urea fertilizers coating agents for slow-release [6-10]. Owing to its innumerable agricultural benefits, the Government of India has legally mandated neem oil coating of all fertilizer urea [11].

Production of neem oil can be increased by converting naturally diploid *A. indica* into polyploid. Also, polyploid plant species are more vigorous than their diploid counterparts. Chromosomal mutation and polyploidy induction are quick methods of plant improvement and colchicine is being used successfully in many trees and crops [12, 13]. The appropriate dose of colchicine leads to the prevention of spindle fiber formation in plant samples which subsequently duplicates chromosome number without cell division and consequently, polyploidy is induced. Colchicine works effectively on different types of plant tissues like seeds, buds, calli, leaves, and nodes and has been effectively used to induce polyploidy in several plant species *viz*., *Miscanthus* and *Populus* species [12, 14, 15]. Colchicine ($C_{22}H_{25}NO_6$), which is known as a successful mitotic inhibitor [16] was used for polyploidy induction of *A. indica* in the present study. Though colchicine has the potential to induce polyploidy, it has a narrow safety window and its overdose leads to hazardous effects [17]. Mitigation of the harmful effect of a chemical depends upon the application of the appropriate concentration of the toxic substance, the method of its application, and also the nature of the test sample [18].

LD_{50} dose or the semi-lethal dose is the concentration of colchicine that kills 50% of the sample in one application and its estimation is important to determine the potential toxicity of the concentration of the chemical [13, 19]. Evaluation of LD_{50} dose of colchicine treatment can be observed through the ocular toxicity method *i.e.,* an easy visual observation, while mathematical methods like the Spearman-Karber method and statistical (graphical) method

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like the Miller-Tainter method accurately quantify the observation. All three methods are being used for the estimation of LD_{50} of potentially toxic chemicals but their efficacy has rarely been compared in plants. Also, genetic variation in neem against the toxic effect of colchicine is not studied yet. In the following study, an LD_{50} dose of colchicine was estimated using three methods; Ocular toxicity, Spearman-Karber, and Miller-Tainter, on calli of three genotypes of *A. indica* under *in vitro* conditions, and their efficacy was compared.

2. Experimental

The leaf explants of 3 genotypes FRIH12, FRIH22 and AFRIC1 were cultured in Murashige-Skoog (MS) medium supplemented with 1 mg/l BAP and IBA in equal concentration. Within 45 days of culture, green calli were obtained and were further subcultured in MS medium supplemented with 1 mg/l benzyl aminopurine (BAP) with ten concentrations of colchicine i.e., (0, 10, 20, 30, 40, 50, 60, 70, 80, 90) mg/l and treatments were repeated in 10 replicates. There is a uniform dose difference of 10 mg/l between two consecutive treatments. Proper safety protocols were maintained while using colchicine [20]. Inoculated cultures in the above treatments were incubated for 45 days at 25± 2°C, 50 μ mol m^{-2} sec^{-1} photosynthetic photon flux density (PPFD), and photoperiod of 16/8 (light/dark). Cultures turning from green to brown within 45 days were considered dead and were noted. The lethality of colchicine doses on inoculated calli cultures was observed and data was recorded. Semi-lethal or LD_{50} treatment was determined by the following methods.

2.1 Ocular toxicity observation method

This is the oldest method to determine lethal doses. In this, dead as well as alive calli cultures were counted against each dose. After the culmination of treatment, the observation was binomial; either dead or alive in each dose per replication (Figure 1). A dose having 5 or more dead calli in 10 replications was considered as 50 % mortality or LD_{50} treatment dose of colchicine.

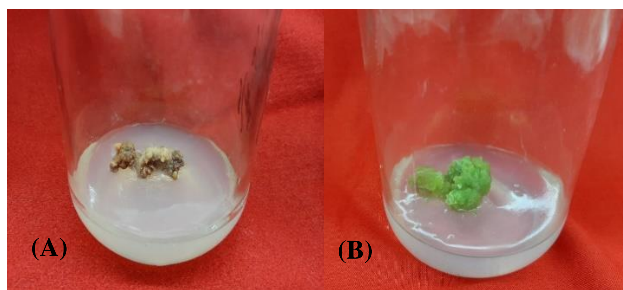

Figure 1: (A) Brown dead callus culture formed on treatment with 90 mg/l colchicine vs (B) green alive callus culture maintained on treatment with 30 mg/l colchicine.

2.2 Spearman-Karber method

The method is as follows [21]:

$$LD_{50} = d - \frac{\Sigma \text{ values of Product } (a \times b)}{\text{number of replicates}}$$

d = the first dose in which 100% lethality is observed,
a = dose difference between two consecutive treatments which is 10 mg/l in this case,
b = mean mortality in each dose

It was determined by taking an average number of dead calluses in preceding and current doses. In the first dose mean mortality (b) is usually nil. The dose difference (a) between the varying doses of colchicine was mentioned as 10 and several dead cultures for each dose were noted according to visual observation. The mean mortality (b) calculated, is the difference between the number of dead cultures in one dose and the number of dead cultures in the next dose. Then the product (a×b) was calculated by multiplying the value of dose difference (a) and mean mortality (b) for each colchicine dose. All the values of Product (a×b) obtained for each dose in a particular genotype were added. This method does not involve any plotting of the dose-response curve.

2.3 Probit analysis by Miller-Tainter

This method is a statistical method of calculating LD_{50} dose where the percentage mortality which is observed is transformed into probit and the values thus procured are plotted against log dose [22]. The log 10 values of each colchicine dose were calculated for each genotype and the percentage of dead cultures was noted down according to visual observation. Corrected mortality was acquired utilizing Abbott's formula and their corresponding probit conversions were obtained from the probit transformation table. The process is shown in Table 1.

3. Results

Semi-lethal dose of colchicine was estimated by observing the survival of calli in the experiment. The observed data were binomial and three methods were employed to determine accurate estimation methods.

3.1 Ocular toxicity observation method

Data on the survival of calli were plotted on a graph, those doses which had 50% or less survival against each genotype were below the LD_{50} line in Figure 2. By this method in genotypes FRIH12, FRIH22 and AFRIC1, LD_{50} of colchicine was 50 mg/l, 60 mg/l and 50 mg/l respectively (Figure 2). The graph depicted that 10 mg/l of colchicine is non-lethal for calli of all genotypes even after 45 days. There was 100 % survival noticed in genotype FRIH22 till 30 mg/l of colchicine. The highest dose (90 mg/l) of colchicine was 100 % lethal in all three genotypes.

3.2 Spearman-Karber method

The result shows the procedure by which the LD_{50} dose of colchicine for each genotype i.e., FRIH12, FRIH22, and AFRIC1 has been estimated (Table 2). The lowest dose of

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Table 1. Calculation of LD_{50} dose by Miller-Tainter method.

Genotypes	Dose	log dose	% Dead	% Correction	Probits
	10	1	0	2.5	3.04
	20	1.30	10	10	3.72
	30	1.48	30	30	4.48
	40	1.60	40	40	4.75
FRIH12	50	1.70	50	50	5.00
	60	1.78	60	60	5.25
	70	1.85	70	70	5.52
	80	1.90	90	90	6.28
	90	1.95	100	97.5	6.96
	10	1	0	2.5	3.04
	20	1.30	0	2.5	3.04
	30	1.48	0	2.5	3.04
	40	1.60	10	10	3.72
FRIH22	50	1.70	30	30	4.48
	60	1.78	50	50	5.00
	70	1.85	80	80	5.84
	80	1.90	80	80	5.84
	90	1.95	100	97.5	6.96
	10	1	0	2.5	3.04
	20	1.30	10	10	3.72
	30	1.48	20	20	4.16
	40	1.60	40	40	4.75
AFRIC1	50	1.70	50	50	5.00
	60	1.78	60	60	5.25
	70	1.85	70	70	5.52
	80	1.90	80	80	5.84
	90	1.95	100	97.5	6.96

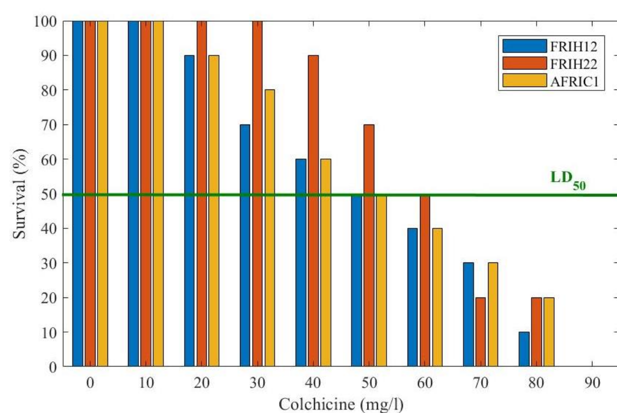

Figure 2: Percentage survival of *A. indica calli* of three genotypes against colchicine doses (mg/l) based on ocular toxicity observation. Percentage survival of genotypes was depicted on Y axis and concentration of colchicine doses was on X axis.

colchicine which killed 100% of cultures was 90 mg/l, Summation (Σ) values of the product for genotypes FRIH12, FRIH22, and AFRIC1 as obtained earlier were 400, 300, and 370, and many replicates were 10, so on putting the values in the Spearman-Karber formula, desired LD_{50} dose of the three genotypes was obtained as 50mg/l, 60 mg/l, and 53 mg/l, respectively.

3.3 Probit method

A regression equation was formed between $\log_{10}$ values of the colchicine concentrations and probit transformations, which gave a value, for a probit of 5 (50 % mortality). In genotypes FRIH12, FRIH22, and AFRIC1, the values obtained were 1.7, 1.78, and 1.7, respectively, as shown in Figure 3. The Antilog calculation of these values gave the semi-lethal dose of colchicine as 50.1 mg/l, 60.3 mg/l, and 50.1 mg/l for the three genotypes respectively.

4. Discussion

According to the ocular toxicity method, the semi-lethal dose of colchicine for neem callus of three genotypes FRIH12, FRIH22, and AFRIC1 is 50 mg/l, 60 mg/l, and 50 mg/l. Spearman-Karber method suggests that the semi-lethal dose of the genotypes is 50mg/l, 60mg/l and 53 mg/l respectively whereas according to the Miller-Tainter method, the semi-lethal dose for the same is 50.1 mg/l, 60.3 mg/l, 50.1 mg/l respectively (Table 3). In this case, the semi-lethal dose in ocular toxicity observation and Spearman-Karber was found to be 50 mg/l for genotype FRIH12 and 60 mg/l for FRIH22. Also, the result was found to be genotype-specific in ocular toxicity observation as FRIH22 gave a higher semi-lethal dose (mean value = 60).

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Table 2. Calculation of semi-lethal dose by Spearman-Karber method

Genotypes	Dose mg/l	Dose diff (a)	No of dead	Mean mortality (b)	Product (a×b)
FRIH12	0	...	-	-	-
	10	10	0	0	0
	20	10	1	0.5	5
	30	10	3	2	20
	40	10	4	3.5	35
	50	10	5	4.5	45
	60	10	6	5.5	55
	70	10	7	6.5	65
	80	10	9	8	80
	90	10	10	9.5	95
FRIH22	0	...	0	…	0
	10	10	0	0	0
	20	10	0	0	0
	30	10	0	0	0
	40	10	1	0.5	5
	50	10	3	2	20
	60	10	5	4	40
	70	10	8	6.5	65
	80	10	8	8	80
	90	10	10	9	90
AFRIC1	0	...	0	…	0
	10	10	0	0	0
	20	10	1	0.5	5
	30	10	2	1.5	15
	40	10	4	2	20
	50	10	5	4.5	45
	60	10	6	5.5	55
	70	10	7	6.5	65
	80	10	8	7.5	75
	90	10	10	9	90

Table 3. Mean and standard deviation (SD) of semi-lethal doses of neem genotypes (FRIH12, FRIH22 and AFRIC1) estimated through ocular toxicity, Spearman-Karber and Miller-Tainter methods

	FRIH12	FRIH22	AFRIC1	Mean ±	SD
Ocular toxicity observation	50	60	50	53.33	5.8
Spearman-Karber method	50	60	53	54.33	5.1
Miller-Tainter	50.1	60.3	50.1	53.5	5.9
Mean	50.03	60.1	51.0		
±SD	0.05	0.173	1.70		

This may be due to the reason that colchicine found difficulty in reacting with the callus tissue of FRIH22. When the three methods used, were compared by taking out the mean of the semi-lethal dose of all genotypes of that particular method, it was noticed that there is a negligible difference amongst the results of the three methods utilized, but the mean of LD_{50} dose taken, of Spearman-Karber method (54.33) varies more than the LD_{50} dose mean of Miller-Tainter method from the mean result of ocular toxicity observation. Also, the Miller-Tainter method gives a semi-lethal dose in decimal value which points out its accuracy.

Though any one of the methods can be used to determine the semi-lethal dose of colchicine for plant species, the Miller-Tainter method is a more reliable method for the determination of a semi-lethal dose of any toxic substance as this is graphically proven [23]. LD_{50} is dependent on many factors such as species, genotypes, age, environment, etc, hence, there are limitations of LD_{50} and results may differ to great extent [24, 25]. Prior knowledge of the LD_{50} dose of colchicine for neem will help in reducing the

amount of time, labor, explant, and colchicine wasted in conducting experiments and hence may help in the application of an appropriate dose of the chemical for induction of polyploidy in *A. indica*.

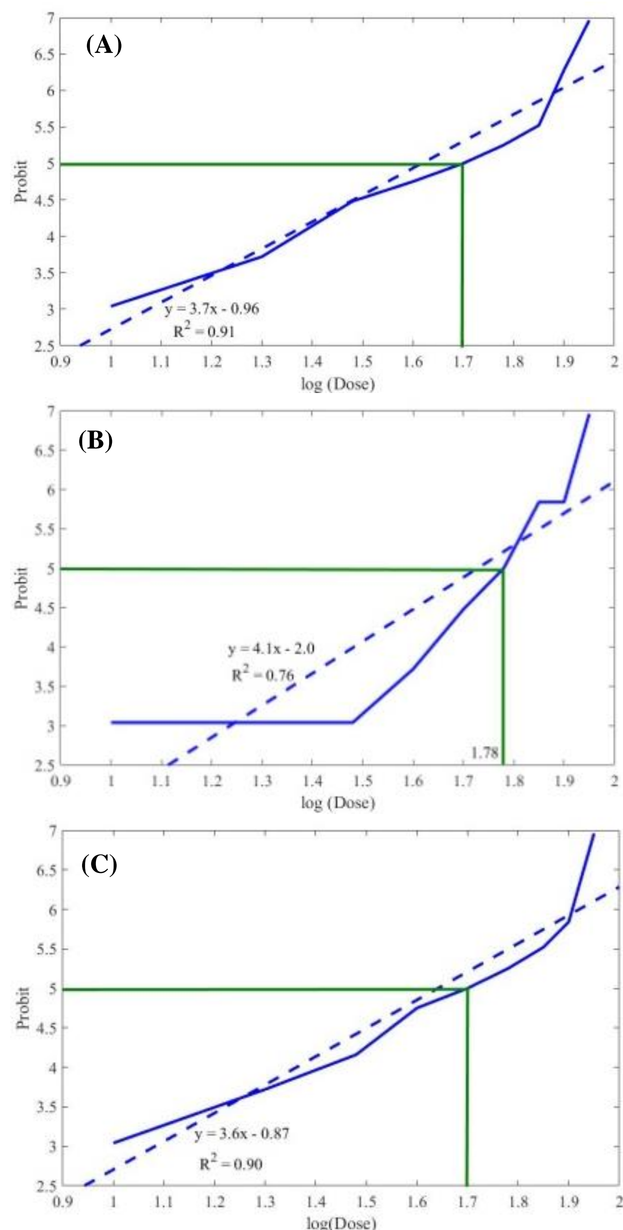

Figure 3: LD_{50} for (A) FRIH 12 (50.1 mg/l), (B) FRIH22 (60.3 mg/l), and (C) AFRIC1 (50.1 mg/l).

5. Conclusion

Polyploid plant species are considered to be better than their diploid counterparts as polyploids provide more biomass and a better yield of oil and useful chemicals. As colchicine has proved to be successful in inducing polyploidy in most tree species using callus tissue, hence, in the present study, *A. indica* has been subjected to colchicine to obtain the desired result. As colchicine is a toxic chemical and usage of excessive concentration can lead to the death of the plant tissue, prior knowledge of semi-lethal dose of colchicine on neem tissue, is important. Hence, the present study involves finding out the semi-lethal dose of colchicine on callus tissue of three genotypes of *A. indica*, before actually applying the chemical to the tissue to induce genetic variation or polyploidy for improvement of neem.

Declarations

Author Contribution: SR, AT, and RS have contributed to the conception of the presented idea for the article; SR did the experiments and data analysis; AT, AK, and SY provided supervision; all the authors have contributed to preparing the manuscript.

Conflict of Interest: The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in TABCJ belongs to the author(s).

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