IN VITRO DEVELOPMENT OF DROUGHT TOLERANT RICE THROUGH POLYETYLENE GLYCOL (PEG) USING EMBRYOGENIC CALLI

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Abstract

Selection for drought resistance is gaining more importance in rice (*Oryza sativa* L.) but selection under natural field conditions is tiresome due to low heritability and time required. Selection in tissue culture is thought to be the preferred way to accelerate selection efficiency. Several parameters such as seed germination percentage, callus induction, shoot regeneration and root induction was studied under drought stress imposed by polyetylene glycol (PEG 6000). Rice varieties Binadhan-19 and Binadhan-17 showed significant callus induction. Callus induction was significantly higher under dark condition (85.2%) than light condition (75.83%). After callus induction, drought stress was created by increased levels of PEG (0%, 5%, 8% and 10%). The highest and the lowest percentage of plant regeneration were found in both varieties at 0% and 10% PEG. The variety Binadhan-19 performed best at 5.0% PEG for shoot regeneration (64%) and root induction (60%) followed by 8% and 10% PEG. At 8% PEG level, shoot regeneration was 56% in Binadhan-19 and 40% in Binadhan-17 and root induction was 40% in Binadhan-19 and 30% in Binadhan-17. At 10% PEG level, shoot regeneration was 40% in Binadhan-19 and 32% in Binadhan-17 and root induction was 30% in Binadhan-19 and 20% in Binadhan-17. The variety Binadhan-19 performed better against drought stress than Binadhan-17. These results emphasize that, selection of drought tolerant rice plants *in vitro* by creating artificial water stress using PEG in culture media is feasible. The information gained from the study could be helpful in developing rice varieties *in vitro* for drought tolerance.

Key Words: Callus Induction, Drought Stress, Plant Regeneration, PEG.

Introduction

Drought is one of the major abiotic stresses that severely affects and reduces the yield and productivity of food crops worldwide up to 70% (Kaur *et al*., 2008; Thakur *et al.*, 2010; Akram *et al.*, 2013). The response of plants to drought stress is complex and involves changes in their morphology, physiology and metabolism. Drought stress is characterized, among others by the reduction of water content, closure of stomata and limitation of gas exchange. Much more extensive loss of water can lead to accumulation of reactive oxygen species (ROS), which disrupts metabolism and cell structure and eventually the enzyme-catalyzed reactions and finally may result in the death of plant (Jaleel *et al*., 2008; Phung *et al*., 2011).

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Rice (*Oryza sativa* L.), the world's most important cereal crop, is the primary source of food and calories for about half of mankind (Khush, 2005). Rice provides as much as 80% of the dietary calories in some Asian countries. The demand for increase in rice production is increasing to meet the needs of increasing world population. The demand for rice is constantly rising in Bangladesh with nearly 2.7 million people being added each year to its population of about 143 million (FAO, 2003). About 77.69% of total cultivable land in Bangladesh is being used for rice production, which contributes 21.22% of total agricultural production and engages about 48% of total agricultural labor forces (BBS, 2007). Rice constitutes the most important economic activity and the primary source of income and employment for more than 100 million households in Asia and Africa (FAO, 2004). The predominantly rice-growing areas in Asia (130 million hectares, more than 85% of the total world rice production) are often threatened by severe abiotic stresses. Bangladesh is facing rice production constraints such as drought, lack of irrigation facilities, flooding, and salinity of soils, coupled with fluctuation of commercial prices.

Binadhan-19 was developed by the Bangladesh Institute of Nuclear Agriculture (BINA), from NERICA-10 through radiation. It is a high yielding and drought tolerant rice variety which can be cultivated both in Aus and T. aman season. Binadhan-19 is long and slender and also cost effective as it requires less irrigation and fertilization. It is a short-duration variety which can be harvested within 90 to 95 days. While other Aus varieties take 110 to 115 days.

Binadhan-17 is a new highly yielding green super rice variety of T. aman developed by Bangladesh Institute of Nuclear Agriculture (BINA). The yield of Binadhan-17 is at least 33 percent higher than that of other varieties of T. aman Rice and production cost is around one-third lower. Binadhan-17 is harvested one-month earlier than other varieties of T. aman allowing cultivation of different winter crops. Polyethylene glycol (PEG) is known to cause osmotic stress *in vitro* which alters the osmotic potential of the cell and hence these serve as useful selection agents for drought tolerance. PEG with molecular mass of 6000 and above are non-ionic, non-toxic, water soluble polymers which do not penetrate intact plant tissues rapidly. It forms hydrogen bonds with water, decreasing the water potential of culture medium and finally inhibits both water and mineral uptake by roots. Thus, such an osmotic agent acts in lowering the water potential in a way similar to soil drying.

Drought is considered as one of the major abiotic stresses limiting rice production worldwide. Therefore, development of drought tolerant rice varieties has been considered as one of the strategies to increase rice production in drought prone areas. The main objective was to study *in vitro* callus induction and regeneration ability of Binadhan-19 and Binadhan-17 under drought stress.

Materials and methods

The experiment was carried out during the period from June to December 2021 in the Tissue culture laboratory at Biotechnology Division in Bangladesh Institute of Nuclear

Agriculture, Mymensingh, Bangladesh. Seeds of two rice varieties viz. Binadhan-17 and Binadhan-19 were collected from Plant Breeding Division, BINA, Mymensingh.

Surface sterilization of mature seed was carried out under laminar air flow cabinet. After collection of seeds following washed by sterilize water for 3 to 5 minutes to reduce the level of surface organism. The floating seeds were discarded. Later, they were rinsed in 70% ethyl alcohol for 2 minutes, followed by washing with sterile distilled water for 3 times. Finally, inside the cabinet, surface disinfection was done with 0.1% $HgCl_2$ for 2 minutes and during this period the sterilant was occasionally agitated. The seeds were then washed 5 times with sterilized distilled water to remove the sterilant. To ensure aseptic condition under *in vitro*, all instruments, glassware and culture media were sterilized by autoclaving with 15 PSI pressure at 121°C for 20 minutes. All inoculation and aseptic manipulations were carried out in a laminar air flow cabinet. The cabinet was exposed to the UV light for 30 minutes before use and cleaned with 70% ethyl alcohol to eliminate the chance of contaminations.

The MS medium formulated by Murashige and Skoog (1962) with vitamins (Duchefa, Netherlands) was used in the study. To test the embryo survivability, MS medium supplemented with NAA at 1.0 mgl^{-1}, BAP and Kinetin at 2.0 mgl^{-1} and different concentrations of (PEG) (6000) (0%, 5%, 8%, 10%). Sterilized mature seeds of two varieties of rice were inoculated into petridish with forceps each containing 25-30 ml MS medium with PEG at different concentrations viz. To = 0% (control/no PEG), T_1 = 5% PEG, T_2 = 8% PEG, T_3 = 10% PEG with three replication. Cultures were maintained at ($25\underline{+}2$) ^{o}C temperature under both light and dark condition. The data on germination percentage, percentage of callus induction, percentage of embryogenic callus induction, percentage of shoot and root regeneration and finally percentage of established plant was recorded.

Statistical Analysis: The recorded data were statistically analyzed using Statistix 10 program and Microsoft Excel wherever applicable. The experiment was arranged in Completely Randomized Design (CRD) with three replications and the collected data were analyzed statistically following analysis of variance (ANOVA) technique and the mean differences were compared by DMNRT (Ducan's Multiple New Range Test) and the ranking was indicated by letters.

Results and discussion

Callus induction capacity from mature embryos

The rice varieties (Binadhan-19 and Binadhan-17) were cultured in MS medium supplemented with different concentrations and combinations of plant growth regulators. The mature embryos were inoculated on MS medium supplemented with 2, 4-D (2.0 mgl^{-1}) to observe the callus formation response. Callus formation invariably occurred from the embryo region of the seeds and was visible within 14 days. There were two different photoperiod conditions. One was under 24 hour dark condition and another one was under 24 hour light using 3000 lux intensity.

Table 1. Effect of dark and light condition on callus induction and embryogenic callus induction of Binadhan-17 and Binadhan-19

Sl. No.	Effect of dark condition				Effect of light condition			
	callus induction (%)		Embryogenic callus induction (%)		callus induction (%)		Embryogenic callus induction (%)	
	Bina dhan-17	Bina dhan-19	Bina dhan-17	Bina dhan-19	Bina dhan-17	Bina dhan-19	Bina dhan-17	Bina dhan-19
1.	83.87	87.09	63.22	67.74	77.41	80.64	52.9	56.77
2.	83.33	86.66	58.78	64	76.67	76.67	49.34	60
3.	82.75	88.27	63.44	75.86	75.86	82.75	52.41	57.93
4.	78.57	89.28	61.42	70	64.28	80	48.57	67.14
5.	85.18	85.92	60.74	68.15	68.14	81.48	51.85	57.77
6.	80.76	90.76	53.84	68.46	63.07	80.76	46.15	63.07
Mean	82.41	87.99	60.22	69.03	70.90	80.38	50.20	60.45

Under dark condition the average percentage of callus induction was obtained in the variety Binadhan-19 (87.99%) which was significantly different from the variety Binadhan-17 (82.41%).

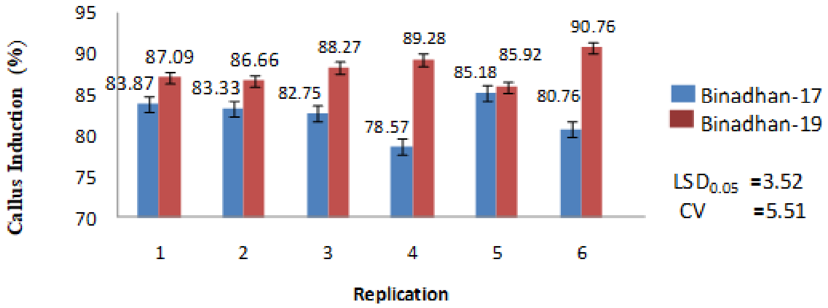

Fig. 1: Effect of dark condition on callus induction (%) of two rice varieties. (Data were recorded after 14 days of inoculation**)**

Under light condition average percentage of callus induction was obtained from the variety Binadhan-19 (80.38%) which was significantly different from the variety Binadhan-17 (70.90%)

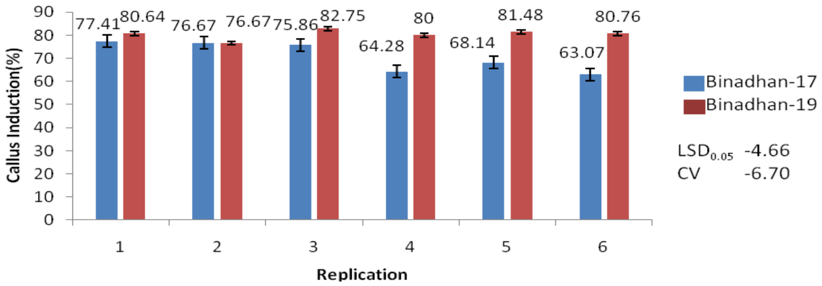

Fig. 2: Effect of light condition on callus initiation (%) of two rice varieties. (Data were recorded after 14 days of inoculation**)**

From the recorded data, it was clearly observed that dark condition was more effective than light condition for callus initiation.

Embryogenic callus induction from mature embryos

The mean squares of varieties for the percentage of callus induction are highly significant indicating the presence of adequate variability between the varieties for this parameter. The average percentage of embryogenic callus induction in dark condition was obtained from the variety Binadhan-19 (69.03%) which was significantly different from Binadhan-17 (60.22%).

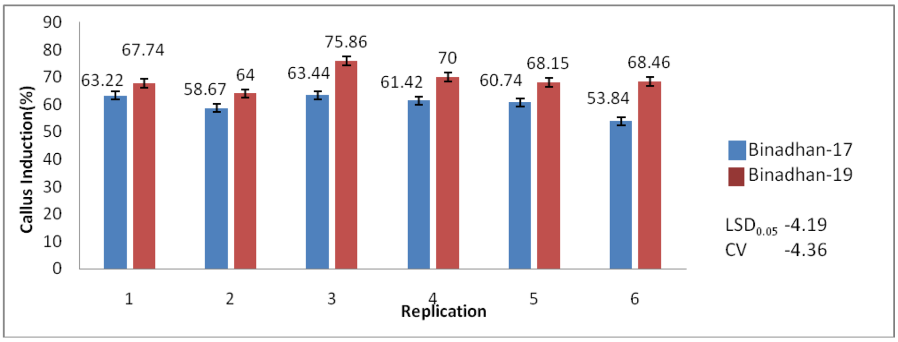

Fig. 3: Effect of interaction between varieties on percentage of embryogenic callus induction in the dark condition.

The higher average percentage of embryogenic callus induction in light condition was obtained from Binadhan-19 (60.45%) which was significantly different from Binadhan-17 (50.20%).

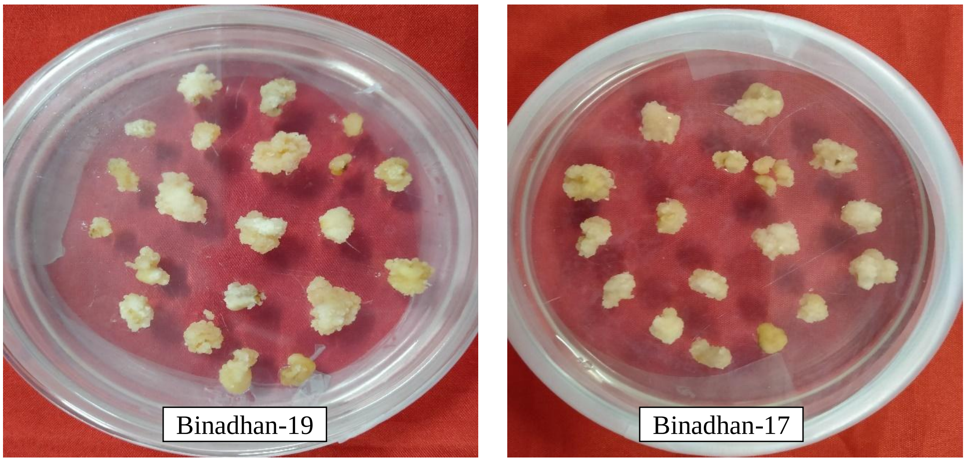

Fig. 4: Embryogenic Callus initiation of two rice varieties cultured on MS medium supplemented with 2, 4-D in dark condition.

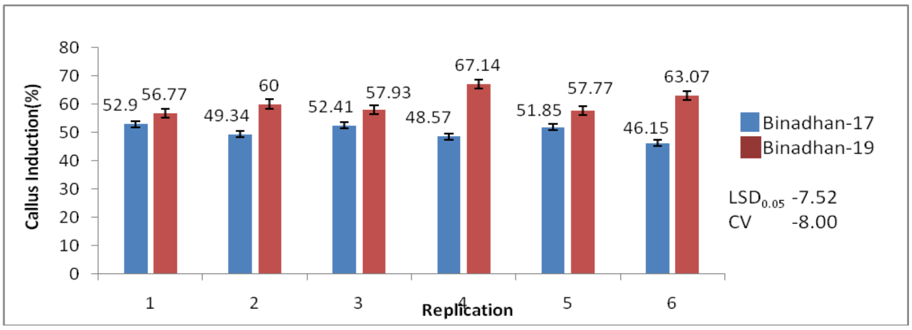

Fig. 5: Effect of interaction between varieties on percentage of embryogenic callus induction in the light condition.

In Vitro shoot regeneration from embryogenic calli response to polyethylene glycol (PEG)

Embryogenic calli of two rice varieties were cultured in MS medium with different plant growth regulators NAA 1.0 mgl^{-1}, BAP and Kn both at 2.0 mgl^{-1}. To induce drought stress, different concentrations of PEG (0%, 5%, 8% and 10%) were added in the culture medium. After 14 days, it was clearly observed that the extent of regeneration ability varied from different PEG concentration. In the two varieties, regeneration percentage was found higher in control. Growth rate and regeneration capacity were decreased with increasing levels of PEG concentration. Among the different concentrations of PEG, The lowest value was recorded for 10% PEG in both the varieties Binadhan-17 (32%) and Binadhan-19 (40%). At 5% PEG, the highest value 48% was recorded in Binadhan-17 and 64%in Binadhan-19.

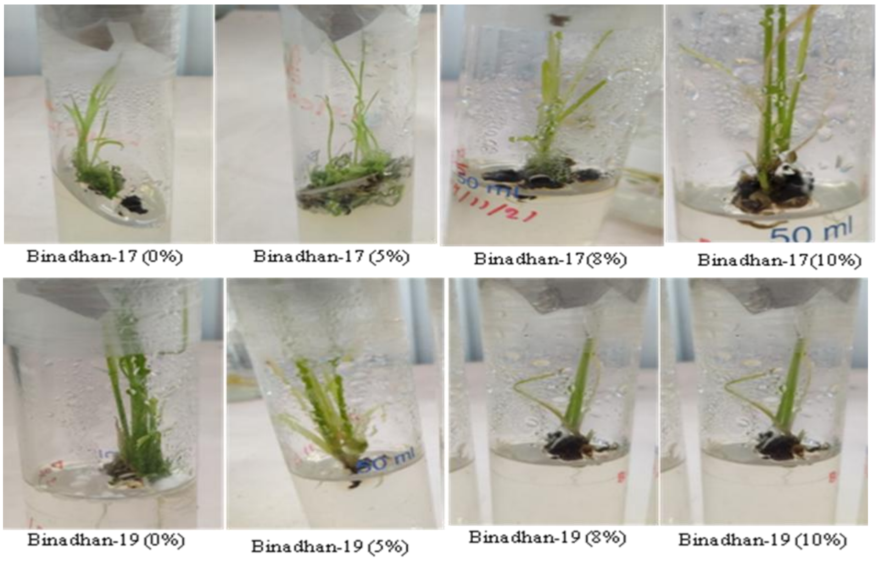

Fig. 6: Shoot regeneration of rice varieties cultured on different concentration of PEG (0%, 5%, 8% and 10%) respectively.

Table 2. Effect of different concentrations of PEG on shoot regeneration ability

Varieties	PEG (%)	No. of calli inoculated	No. of calli showing shoot initiation	Shoot regeneration %
Binadhan-17	$T_0 = 0$	25	19	76
	$T_1 = 5$	25	12	48
	$T_2 = 8$	25	10	40
	$T_3 = 10$	25	8	32
Binadhan-19	$T_0 = 0$	25	20	80
	$T_1 = 5$	25	16	64
	$T_2 = 8$	25	14	56
	$T_3 = 10$	25	10	40

Here, T_0 = MS Media + 0% PEG + NAA 1.0 mgl^{-1}, BAP & Kin at 2.O mgl^{-1}
T_1 = MS Media + 5% PEG + NAA 1.0 mgl^{-1}, BAP & Kin at 2.0 mgl^{-1}
T_2 = MS Media + 8% PEG + NAA 1.0 mgl^{-1}, BAP & Kin at 2.0 mgl^{-1}
T_3 = MS Media +10% PEG + NAA 1.0 mgl^{-1}, BAP & Kin at 2.0 mgl^{-1}

In Vitro root induction from regenerated shoot in presence of Polyethylene glycol (PEG)

Half strength of MS media supplemented with different concentrations of PEG (0%, 5%, 8% and 10%) were used to see the rooting response of the regenerated shoots. MS medium supplemented with different concentrations of PEG and no growth regulator hormones was found most effective for root induction. The lowest value was recorded for the concentration of 10% PEG in both the varieties Binadhan-17 (20%) and Binadhan-19 (30%) respectively. The highest value at 5% PEG was recorded in Binadhan-17 (50%) and Binadhan-19 (60%) respectively.

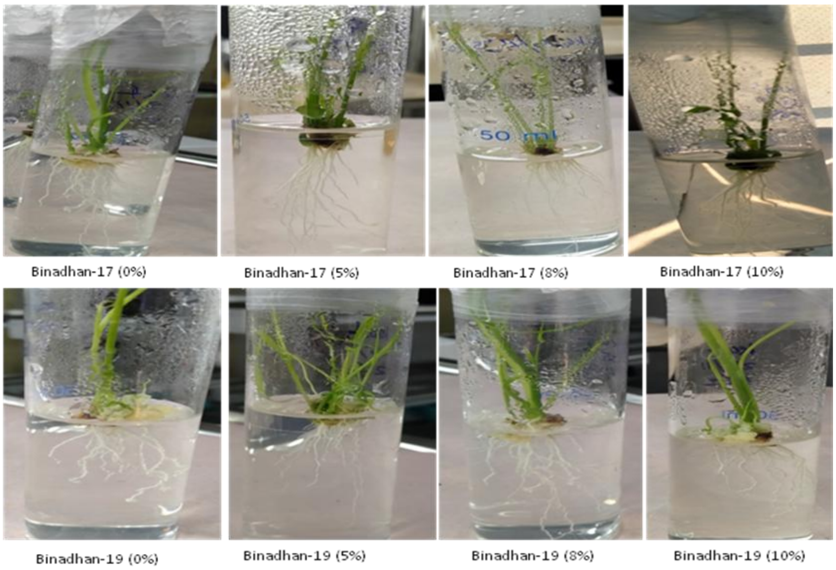

Fig.7: Root induction of two rice varieties cultured on half strength of MS medium supplemented with different concentrations of PEG (0%, 5%, 8% and 10%) respectively.

Table 3. Effect of MS medium (Half strength) supplemented with different concentration of PEG on root induction

Varieties	PEG (%)	No. of shoot inoculated	No. of shoot showing root induction	Root induction %
Binadhan-17	$T_0 = 0$	10	7	70
	$T_1 = 5$	10	5	50
	$T_2 = 8$	10	3	30
	$T_3 = 10$	10	2	20
Binadhan-19	$T_0 = 0$	10	7	70
	$T_1 = 5$	10	6	60
	$T_2 = 8$	10	4	40
	$T_3 = 10$	10	3	30

Here, T_0 (Control) = Half strength of MS media + 0% PEG
T_1 = Half strength of MS media + 5% PEG
T_2 = Half strength of MS media + 8% PEG
T_3 = Half strength of MS media + 10% PEG

Establishment of plants

The small plantlets that attained good shoot development and produced sufficient roots were transfer to pots containing 50% soilrite (1:1:1 ratio of vermiculite, perlite and Sphagnum moss) mixed with soil for hardening. Excess agar around the roots was washed off by tap water to prevent microbial infection. Then the pots were kept in a humid chamber for 3-5 days in the culture room under 16h light and 8h dark cycle at 28^{o}C and then in green house at (28$\pm$2)^{o}C. Establishment of regenerated plants lead to regeneration of a complete plant on the medium. When the plants grew up to a height of above 10cm and sufficient roots were proliferated, those were transferred to the earthen pot. Tillering capacity and survival rate of plants in the pots were satisfactory. Many studies suggest that MS medium supplemented with just 2, 4-D can produce more calli (Shahsavari *et al*., 2010). However, Mohd Din *et al*. (2016) found high level of callus induction in medium supplemented with 2, 4-D and combination of BAP or NAA. Embryogenic callus grown on MS medium supplemented with four different concentrations of PEG viz. 0% (control), 5%, 8% and 10% PEG, created different osmotic stress condition on the media. Wani *et al*. (2010) showed both reduced callus induction and shoot regeneration of rice varieties PAU 201 and PR116 in medium supplement with different concentrations of PEG. Tripathy (2015) also found similar reduction in regeneration frequency with increased concentration of PEG in upland rice. Further, variety depended responses with PEG have also been observed. Akter *et al*. (2016) found that rice variety Binadhan-10 performed the best against PEG compared to Binadhan-4, Binadhan-5, Binadhan-6 and Iratom-24.In this study, under difference PEG levels Binadhan-19 performed better than Binadhan-17. Shoot regeneration and root induction was higher in Binadhan-19 than Binadhan-17. Both the varieties show reduction in callus induction with increased levels of PEG. The highest percentage of callus induction was recorded in Binadhan-19 under control condition (0% PEG). Considering all the parameters, the two varieties when raised *in vitro* up to the seedling stage without any drought stress (0% PEG), Binadhan-19 showed better performance than Binadhan-17. Similarly, the variety Binadhan-19 performed better at 5% concentration of PEG under drought stress. These findings suggested that the variety Binadhan-19 could be considered as more tolerant against drought condition than Binadhan-17.

Conclusion

Crop genetic improvement for drought stress at the molecular and physiological level is very complex and challenging. The results of this study indicate that, the two rice varieties Binadhan-17 and Binadhan-19 have good callus induction ability as well as inherent tolerance to drought. We suggest that, *in vitro* screening with the induction of chemical drought by using PEG 6000 to modulate drought tolerance would be a feasible strategy to develop drought tolerant lines of rice. The above study will be the base line for future screening experiments.

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