

Effects of graphene oxide nanosheet on germination and seedling attributes on *tagetes erecta* l. And *calendula officinalis* l.

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Abstract: In recent times, nanotechnology is a branch of precision farming and sustainable agriculture where it expresses all sides of development for better utilization of resources in agriculture. Graphene oxide (GO) nanosheet is a two-dimensional crystal structure formed by a flat monolayer of sp² hybridized carbon atoms arranged in a hexagonal arrangement. Marigold (*Tagetes erecta* L.) and Calendula (*Calendula officinalis* L.) are commercially explored flower crops of the family Asteraceae. These flower species contain a sufficient amount of active compounds, used in various commercial sectors. Graphene oxide nanosheets were produced by the commercial chemical method. Characterization of GO is done on powder X-ray diffraction (XRD), Fourier transforms infrared (FTIR) & Scanning electron microscopy (SEM). Seeds were collected from recognized sources and after surface sterilization; seeds were treated with GO nano solution with different concentrations (0, 20,40,60,80,100,120 and 140mg/l respectively). The conducted experiment found that GO in the concentration range from 40 to 80 mg/l (T₂ to T₄) gives positive results in the maximum of all aspects in both the annuals. The maximum root length of the marigold was recorded in T₂ (5.91cm) and shoot length in T₅ with 4.63cm. The coefficient of the velocity of germination (CVG) for calendula was observed maximum in T₃ (32.17) and in marigold T₂ (46.34). In GO treated seeds were having a dense rooting system with good seedling growth, early germination rate also observed in treated seeds in comparison to control.

Key words: Graphene Oxide; Germination; Growth; FTIR; SEM; XRD

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1. Introduction

Nanotechnology is a branch of precision farming where it is exploring its all sides for the development of sustainable agriculture. Nano particles have only one of its kind physicochemical properties and the potential to boost up the plant metabolism (Giraldo *et al.* 2014). According to (Torney *et al.* 2007) engineered nanoparticles are capable to enter into plants cells and leaves, and also can transport DNA and chemicals keen on plant

cells, can also arouse plant growth. Nano particles when applied to the plants can help out them to cope up the harsh conditions by releasing the ROS enzyme (Reactive Oxygen Species). From the resent study it has been found that application of engineered nanomaterials to agricultural sciences has been particularly active and exciting (Lin and Xing 2007, Prasad *et al.* 2012). Under given abiotic stress condition, nano-SiO₂ augments seed germination. SiO₂NPs improves and ignite the photosynthetic rate by synthesis of

photosynthetic pigments and getting better activity of carbonic anhydrase, as reported by (Siddiqui *et al.* 2014 and Xie *et al.* 2012). Xie *et al.* 2011 reported from their study that, it also boost up the plant growth and development by mounting gas exchange and also enhance few parameters, such as net photosynthetic rate, photochemical efficiency, electron transport rate. Zheng *et al.* (2005) observed the ability of nano-TiO₂ from their experiment on the growth of aged spinach seeds and found that the plant physiological parameters like plant dry weight appreciably increases due to the augmented formation of photosynthetic pigments and few biochemical activities. Hong *et al.* (2005) found from their investigation that TiO₂NPs take care of the chloroplast from unnecessary light by augmenting and modify the activity of antioxidant enzymes, such as superoxide dismutase. From one conducted experiment it has been found that, exogenous application of TiO₂NPs positively modifying net photosynthetic rate, also improves transpiration rate in plants, which is stated by Qi *et al.* 2013. From the recent study it has been found that function of engineered and artificially made nanomaterials to agricultural sciences and plant science has been particularly exciting (Lin and Xing 2007; Prasad *et al.* 2012). Lei *et al.* (2007) reported the efficiency of nano-anatase, which promoted strongly whole chain electron transport and photoreduction activity of photosystem II in plant physiology. In the context of carbon nano tubes, graphene is an ultra-thin carbon material with similar chemical and physical properties (Allen *et al.* 2010) and also have massive potentials and ability for biological applications suggested by Dutta and Pati, (2010). On the other hand, Nair *et al.* (2012) found that graphene-treated rice seedlings are healthier with well-developed root and shoot systems as in relation to controls. Zhang *et al.* (2015) justified that Graphene was found to penetrate seed husks that might facilitate water uptake, resulting in faster germination and higher germination rates. Similar study was reported by Khodakovskaya *et al.* (2009), observed that

carbon nano tubes penetrate husk of tomato seeds and amplify the rate of seed germination greatly. Graphene oxide (GO) nanosheet is a two dimensional crystal structure formed by a flat monolayer of sp² hybridized carbon atoms arranged in a hexagonal arrangement, technically it's 2D allotropes of carbon, one which has honeycomb lattice structure, Graphene oxides nanosheet electronic and optical properties enable graphene oxide nanosheet to be used in many field (Geim and Novoselov, 2007). The major concern about graphene oxide is mainly focused on its chemical structure, electronic properties, reduction reaction and chemical functionalization these are different kinds of oxygen species and bonding to carbon in graphene layer, such as epoxy, hydroxy carbonyl & carboxylic groups (Kumar *et al.* 2013). The application fields of graphene oxides are mainly focused on sensor (Robinson *et al.* 2008, Lin *et al.* 2010), efficient catalyst, as a supercapacitor (Liu *et al.* 2010), conducting materials (Zou *et al.* 2009, Lu *et al.* 2010) and as a gas storage medium. Marigold (*Tagetes erecta* L.) and Calendula (*Calendula officinalis* L.) is commercially exploited flower crops belong to family Asteraceae. These flowers are native to Mexico and Mediterranean region respectively. These species has been cultivated since India times for its purposed general medicinal qualities and today both flowers are grown for medicinal and herbal (Matic *et al.* 2012, Mohammad and Kashani 2012, Preethi and Kuttan 2009), and ornamental uses (Clark *et al.* 2010, Warner and Erwin 2005). These flowers species contains a sufficient amount of active substances or compounds, such as: saponosides, flavonides, sterols, polysaccharides, sesquiterpene lactones along with essential oils, because of only this reason they are exploited in food industries as food colorant and supplements. Till now no such research has been conducted on the escalation of these secondary metabolites of these crops with any precision farming such as nano technology.

2. Materials and methods

Experiment was conducted in collaboration between University of Kalyani, Department of Chemistry and Department of Floriculture and Landscape Architecture, BCKV during the period of one year (2018-2019).

GO Preparation and Characterization

Experimental: Materials

Natural Graphite powder (200 mes) of spectroscopic purity was purchased from Gupta Electric Manufacturing Co. (Delhi, India). Conc. H_2SO_4 (98%), H_3PO_4 (85%), and $NaNO_3$ were purchased from Sigma-Aldrich and hydrogen peroxide (H_2O_2 , 30%) and $KMnO_4$ (99%) were purchased from Shijiazhuang Kunmal Chemical Technology Co. (China). All reagents were of AR grade, and were used as received without further purification.

Synthesis of Graphene Oxide nanosheet (GO)

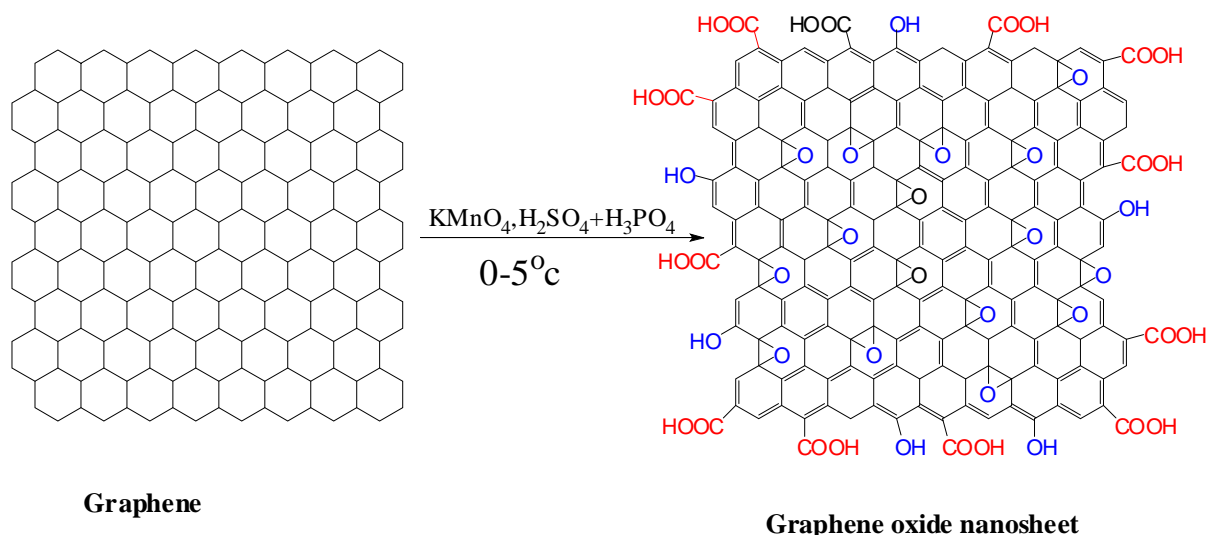
Graphene oxide was synthesized by modified Hummer's method through oxidation of graphite powder. The method of preparation is briefly as follows. Graphite powder (1.5 g) and $NaNO_3$ (0.4g) were mixed in 45 ml of Conc. H_2SO_4 (98%) and 15 ml of H_3PO_4 (85%) in a 500 ml round bottom flask kept under ice bath (0-5 °C) with continuous stirring. The mixture was stirred for 35 minutes at this temperature and potassium permanganate (7.5 g) was added to the suspension very slowly. The rate of addition was carefully controlled to keep the reaction temperature lower than 11-19°C. The mixture was then diluted with very slow addition of

120 ml water and kept under stirring for 1.20 hrs. The ice bath was then removed, and the mixture was stirred at 35°C for 2 hrs. The mixture was then refluxed at

85°C for 10-25 min. After 15 min, the temperature was changed to 40°C, yielding a brown coloured solution. After another 25 min, the temperature of the mixture was changed to 28°C, and maintained at this temperature for 1.00 hrs. The solution was finally treated with 35 ml H_2O_2 whereby its colour changed to bright yellow. 260 ml of water was taken separately in two separate beakers and equal amount of solution prepared was added to it with stirring for 2.50 hr. It was then kept without stirring for 2-3.5 hrs, allowing the larger particles to settle at the bottom and supernatant filtered. The filtrate was washed repeatedly after centrifugation with (5-10) % HCl and then with deionized (DI) water several times until it forms a gel like substance (at neutral pH). The gel like substance was vacuum dried at 50-65°C for more than 3.5 hrs to obtain GO powder (shown in scheme 1).

Characterization

The powder X-ray diffraction (XRD) of the sample was carried out on a Rigaku-MiniFlex 600 X-ray diffractometer with $CuK\alpha$ source ($\lambda=1.5418 \text{ \AA}$) at room temperature. Fourier transform infrared (FTIR) spectra were obtained with Shimadzu FTIR-8300 spectrophotometer. Scanning electron microscopy (SEM) was performed on a ZEISS, EVO40 microscope. Raman spectrum was obtained with a Thermo Nicolet FT-Raman spectrometer.



Scheme 1- Chemical structure of graphene oxide

Seeds Treatments and seeding observation

Experiment was performed in PG lab of Department of Floriculture and Landscape Architecture, Bidhan Chandra Krishi Vishwavidyalaya, Mohanpur, West Bengal. Nano particle Graphene oxide is synthesized and characterized in Department of Chemistry, University of Kalyani, and West Bengal. Seeds were collected from research farm, Mondouri, Kalyani. Proper seed treatment protocol was adopted, where seeds were immersed in a 10% sodium hypochlorite solution for 10 min for disinfection purpose and then they were washed three times with deionized water. Then, disinfected seeds were next soaked in suspensions solution with different concentrations of GO (20, 40, 60, 80, 100, 120 and 140 mg/l). After that, seeds were placed onto filter papers placed in 10cm Petri dishes test unit along with 5 mL of respective concentration of GO, with 10 seeds per dish, then placed in a dark growth chamber under a constant temperature of 25°C.

From the 1st day, after 24 hrs interval germination parameters were observed and recorded separately like, GI- germination index, GRI- germination rate index, MGT- mean germination time, FGP- final germination percentage %, CVG- coefficient of velocity of germination, G3- germination % at 3rd day.

After continuous growth for 7 days in the water and different concentrations of GO, the Marigold (*Tagetes erecta* L.) and Calendula (*Calendula officinalis* L.) seedlings were gently cleaned with water to remove the remaining dirt particles and dry them out on filter paper. Then, MSD- marigold shoots dry weight (gm.), MRD- marigold roots dry weight (gm.), CSD- calendula shoots dry weight (gm.), CRD- calendula roots dry weight (gm.), CRL- calendula shoot length (cm), CRL- calendula root length (cm), MRL- marigold root length (cm), MSL- marigold shoot length (cm) of untreated and treated plants were monitored. All experiments were performed using a completely randomized design with three replicates per treatment.

Data were expressed as mean SD of three experiments in triplicate. Error bars represented the standard deviation of the mean. Statistical comparisons were performed by analysis of variances (ANOVA). The values of p (<0.05 & <0.01) were considered to be statistically significant (*) and highly significant (**) respectively.

Results and discussion

Characterization of GO

SEM study

The Fig. 1 (a, b) are shows the scanning electron microscope of synthesised graphene

oxide nano-sheet materials. These images analysed the morphology of nanostructural shape and size of sheets. SEM of materials shows regular grooved structure, probably from ordering of sheet like structures of graphene oxide nanoflakes or graphene oxide nano-sheet. These images are also observed that the agglomerated sheets of layered type of structure. While the scanning electron microscope image clearly predicated the synthesised materials are graphene oxide nanosheet.

XRD study

Powder x-ray diffraction (p XRD) data of the synthesized graphene oxide nano-sheet taken at room temperature is shown in Fig. 2 below. The characteristic peaks at 2θ value of 10.37° correspond to the crystal faces of (001) planes of pure graphene oxide nano-sheet (JCPDS, GO file no. 75-2078). Powder x-ray diffraction analysis was done to know the synthesized samples graphene oxide nanosheet.

FT-IR spectra study

The FT-IR spectra of graphene oxide (GO) is shown in Fig. 3 below. The peak at 1714 cm^{-1} corresponding to $\nu(\text{C}=\text{O})$ of $-\text{COOH}$ on the GO. The peak at 1601 cm^{-1} corresponding to $\nu(\text{C}=\text{C})$ of aromatic π bonds. The peak at 1047 cm^{-1} corresponds to $\nu(\text{C}-\text{O})$ of epoxy bonds.

Effects of GO on germination parameters of calendula and marigold seeds

As per the conducted experiment, mean germination (days) is found less in T_3 followed by T_2 (3.09 and 3.15 respectively). Germination rate index (%/days) is more effective in T_2 with 36.17 and followed by T_4 (35.33) and T_3 (35.12). Final germination percentage was recorded on 7th day from the experiment started, which is recorded maximum in T_3 and T_4 with 98.33% followed by T_2 with 96.67%. Under the study of germination test of calendula seeds treated with graphene oxide (GO), CVG is observed maximum in T_3 (32.17) which are nearby to T_2 and T_4 (31.91 and 31.83 respectively).

Germination on 3rd day, noted more in T_4 (98.33%) than the rest treatment. Conducted experiment reported that calendula seeds on control are presented less value of preferences as compared to the other data. In some treatment above a particular concentration, germination parameter shows negative impacts over the seeds as shown in Table 1. According to Lee *et al.* (2013), particle size of nano material sometime affects the germination, like smaller the size more absorption rate and vice-versa. Villagarcia *et al.* 2012 and Tiwari *et al.* 2014 stated that MWCTs induce the water and essential Ca and Fe nutrients uptake efficiency that could augment the seed germination.

In the aspects of marigold, from the Table 1, germination parameters were recorded separately after the seed treatment in marigold. Similarly with the calendula, marigold also shows positive results with seeds treated with GO. Germination index (GI) recorded maximum in T_2 with 76.67 followed by T_4 and T_3 (75.33 and 73.47 respectively). Germination rate index (GRI) is observed minimum in T_7 (32.94) which shows that high concentration of GO, germination of seeds were affected, whereas T_2 recorded maximum value with 49.55. More values of mean germination time, shows slow germination rate of the seeds. Mean germination time (MGT) noted minimum in T_2 (2.15days) followed by T_4 and T_3 (2.25 and 2.29 days respectively), which proves that the GO at 40 to 80mg/l concentration leads to fast germination of seeds. Final germination percentage (FGP) observed 100% in T_1 , T_2 and T_4 . Coefficient of velocity of germination (CVG) is more in T_2 (46.34) and it is recorded less in T_7 (27.85) at high concentration of Graphene oxide (GO). Germination percentage at 3rd day recorded maximum in T_4 followed by T_3 and T_2 . Khodakovskaya *et al.* (2009) observed more germination rate and other desirable parameters in vegetable seeds treated with GO.

Effects of GO on seedling parameters of calendula and marigold

In calendula the effects are par with marigold parameters in some aspects (Table 2). Shoot dry weight value is recorded maximum in T₂ (0.0188g) followed by T₅ (0.016g). In the context of root dry weight the value is 0.0994g in T₃ with maximum and 0.0166g in T₀ in control with minimum value respectively. After a week of transplanting the seeding parameters of calendula were recorded and noted separately (Fig-4). Calendula root length is observed maximum in T₂ (5.86cm) followed by T₄ and T₅ (4.4cm and 5.6cm respectively). Shoot length of the treated and untreated calendula seedling showed higher value in T₄ with 3.63cm and lower in T₀ control with 0.13cm. According to previous studies done by various researchers, the effects of nanomaterials on plants depend on the characteristics of the nanomaterials and the xylem anatomy within plants, which affect the absorption rate (Begum et al. 2011, Cañas et al. 2008). Smirnova *et al.* 2012 found from their study that MWCNTs pick up the root and stem growth and dehydrogenase activity may be due to primary uptake and accumulation of MWCNTs by roots followed by the translocation from roots to leaves, which could encourage genes expression (Khodakovskaya *et al.* 2012, Wang *et al.* 2012a).

After the seed germination, when the seeds were transplanted in the germination tray to observe the seedling health. These two flower crops marigold and calendula were planted separately to observe the respective seedling parameters. From the presented Table (2), dry weight of the marigold shoot was recorded maximum in T₄ (0.0064g) followed by T₂ with 0.0052g, while untreated seedlings recorded less value. Similarly, Root dry weight of marigold is observed maximum in T₄ (0.0193g) followed by T₃ (0.0072g). In the context of marigold root length, which showed maximum value in T₄ (6cm) followed by T₃ and T₂ (4.50cm and 5.91cm respectively). Shoot length of marigold seedling is observed high in T₅ (4.36cm) and low in T₀ control with 0.27cm (Fig. 5). Begum *et al.* (2011) reported that because of differences in root systems, the response of some vegetables crops like,

cabbage, tomato, red spinach and lettuce over graphene vary. Khodakovskaya *et al.* (2013) and Husen and Siddiqi, (2014) observed that Sometime high concentrations of graphene inhibited the growth of some species, but had no significant effect on some. This is due to the difference in the uptake of nanomaterials by distinctive xylem structures.

3. Conclusion

From the conducted study it can be concluded that graphene oxide nano sheet really proves beneficial in the context of seed germination and seed parameters of winter annuals (*Tagetes erecta* L.) and (*Calendula officinalis* L.). Seed germination is recorded fast in the seeds treated with GO as compared to control. Seedlings growth was also healthy in treated seeds. Graphene generally trigger the metabolic pathway for fastening the growth and development. Profuse rooting and good shoot/root length was observed in GO treated seeds. At the concentration of 40-80 mg/l GO promote the healthy seedling growth, but at very much high concentration it may restrict the plant growth. GO is one of the good promoting agents for plant growth, it should be explore more in the field of agriculture.

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Table 1. Germination test data of marigold and calendula seeds treated with various concentrations of Graphene oxide Nano particles

Treatment	Germination Index		Germination Rate Index (%/Day)		Mean Germination Time (Days)		Final germination At 7 th Day (%)		Coefficient of Velocity of Germination		Germination % At 3rd Day In Marigold	
	marigold	calendula	marigold	calendula	marigold	calendula	marigold	calendula	marigold	calendula	marigold	calendula
T ₀ (control)	60.33 g	3.42 c	38.03 g	24.67 f	2.93 b	41.08 de	91.67 b	20.38 e	32.89 f	88.33 c	63.33 g	63.33 g
T ₁ (20 mg/l)	71.67 d	3.73 d	45.30 d	25.33 f	2.48 c	40.33 e	100.00 a	25.81 d	40.88 e	93.33abc	85.00bc	73.33 e
T ₂ (40 mg/l)	76.67 a	3.15 a	49.55 a	36.17 a	2.15 f	57.33 a	100.00 a	31.91 a	46.34 a	96.67ab	90.00ab	83.33 c
T ₃ (60mg/l)	73.67 c	3.09 d	48.03 b	35.12 b	2.29 de	58.33 a	98.33ab	32.17 a	43.32 c	98.33 a	83.33 cd	93.33 b
T ₄ (80mg/l)	75.33 b	3.12 d	46.17 c	35.33ab	2.25 e	57.08 a	100.00 a	31.83 a	44.63 b	98.33 b	93.33 a	98.33 a
T ₅ (100mg/l)	63.67 e	3.41 d	40.72 e	31.33 c	2.37 d	49.04 b	93.33ab	32.02 a	42.25 d	90.00bc	78.33 de	78.33 d
T ₆ (120mg/l)	61.67 f	3.45bc	39.22 f	29.04 d	2.47 c	46.08 c	93.33ab	29.20 b	40.32 e	88.33 c	73.33ef	73.33 e
T ₇ (140mg/l)	54.33 h	3.64ab	32.94 h	27.47 e	3.60 a	42.15 d	73.33 c	27.47 c	27.85 g	86.67 c	68.33fg	68.33 f
SD	8.22	0.24	5.69	4.62	0.48	7.70	8.94	4.18	6.28	4.80	10.50	12.08
Standard error	2.91	0.08	2.01	1.63	0.18	2.72	3.16	1.48	2.22	1.70	3.71	4.27

Table 2. Morphological variations in calendula and marigold due application of Graphene oxide Nano particles

Treatments	(1 Week)- Root Length (cm)		(1 Week)-Shoot Length (cm)		Dry weight of shoot/seedling (g)		Weight of root/ seedling (g)	
	calendula	marigold	calendula	marigold	calendula	marigold	calendula	marigold
T ₀ (control)	1.67 d	1.03 e	0.13 g	0.27 f	0.0094 e	0.0032 e	0.0166 f	0.0044 e
T ₁ (20 mg/l)	1.2 e	1.17 e	0.53 f	1.03 e	0.0118 d	0.0034 d	0.0704 c	0.005 d
T ₂ (40 mg/l)	5.86 a	5.91 a	1.07 d	3.3 b	0.0188 a	0.0052 b	0.0706 c	0.0042 f
T ₃ (60mg/l)	2.97 c	3.00 d	0.83 e	2.93 c	0.0138 c	0.0042 c	0.0994 a	0.0072 b
T ₄ (80mg/l)	4.4 b	4.50 b	3.63 a	3.17 bc	0.0142 c	0.0064 a	0.0786 b	0.0192 a
T ₅ (100mg/l)	5.6 a	6.00 a	2.07 c	4.36 a	0.016 b	0.0036 d	0.0594 d	0.0052 d
T ₆ (120mg/l)	4.23 b	4.13 c	2.61 b	3.07 bc	0.012 d	0.0026 f	0.0594 d	0.0058 c
T ₇ (140mg/l)	4.37 b	4.27 c	2.11 c	2.53 d	0.0124 d	0.0036 d	0.0434 e	0.0042 f
SD	1.70453	1.90198	1.081691	1.213707	0.00288	0.00123	0.0246	0.00507
Standard error	0.602642	0.672451	0.382436	0.42911	0.001018	0.00043	0.0087	0.001792

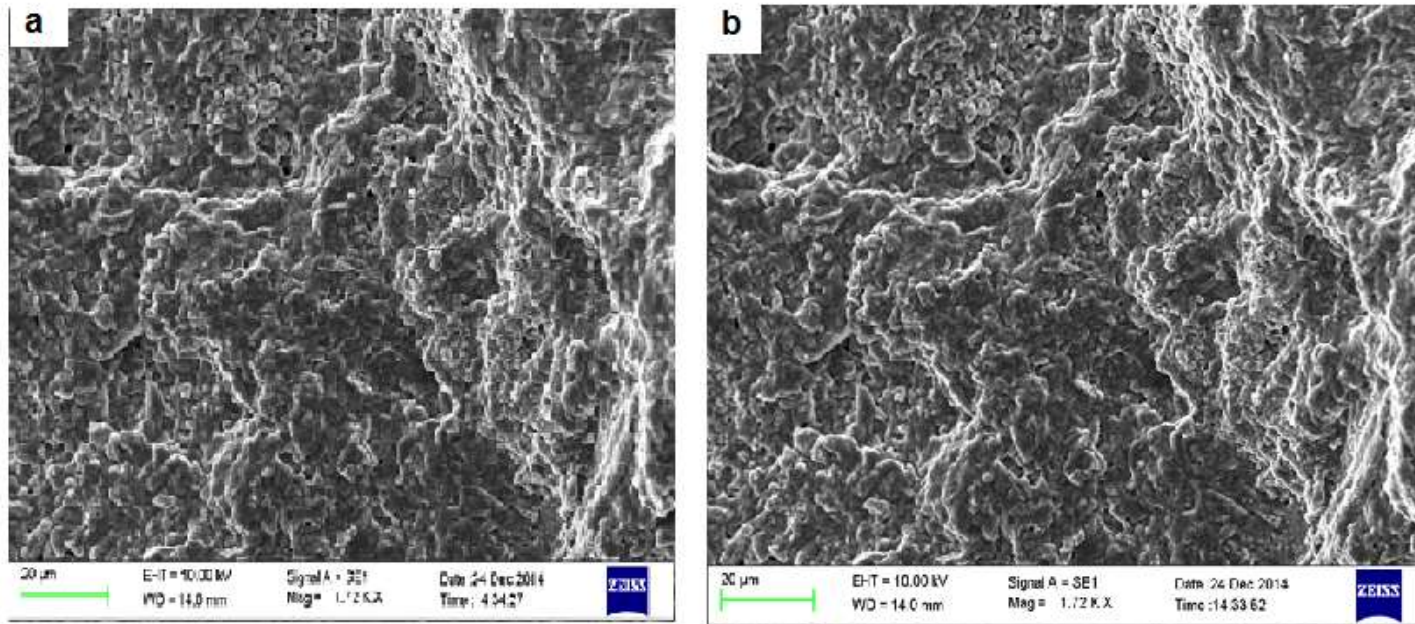


Fig. 1 (a, b) SEM image of synthesized graphene oxide nano-sheet

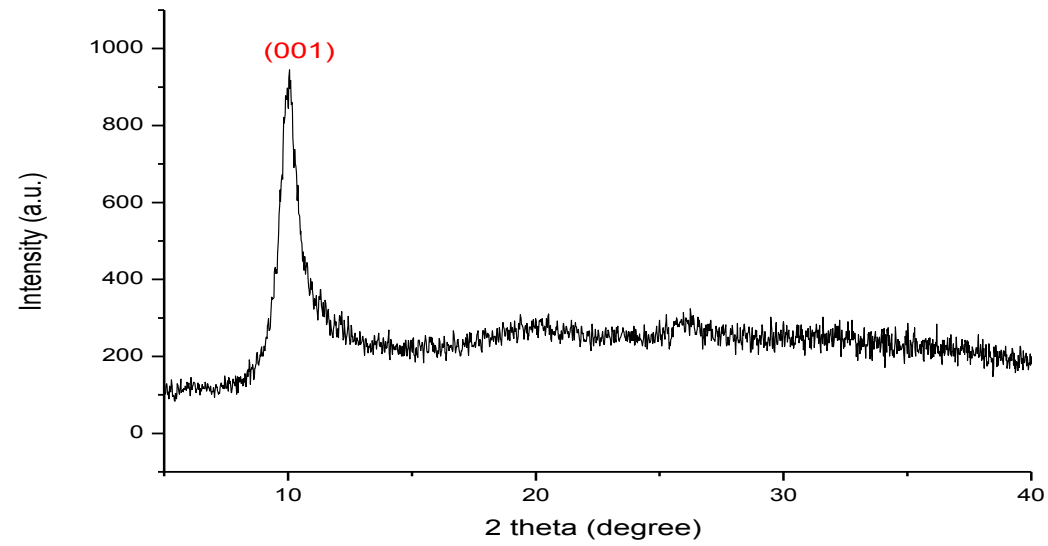


Fig.2. Powder X-ray diffraction (pXRD) data of the synthesized graphene oxide nano-sheet

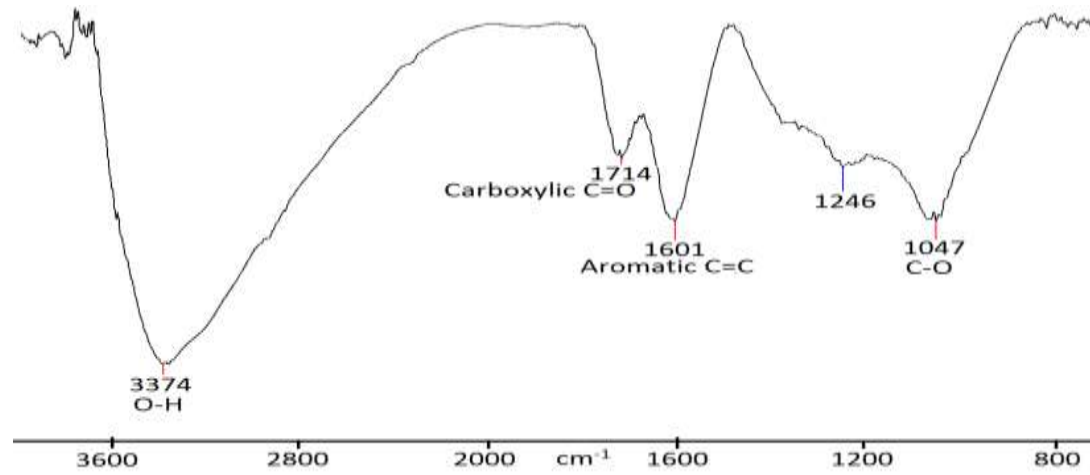


Fig.3. FTIR spectra of graphene oxide nanosheet

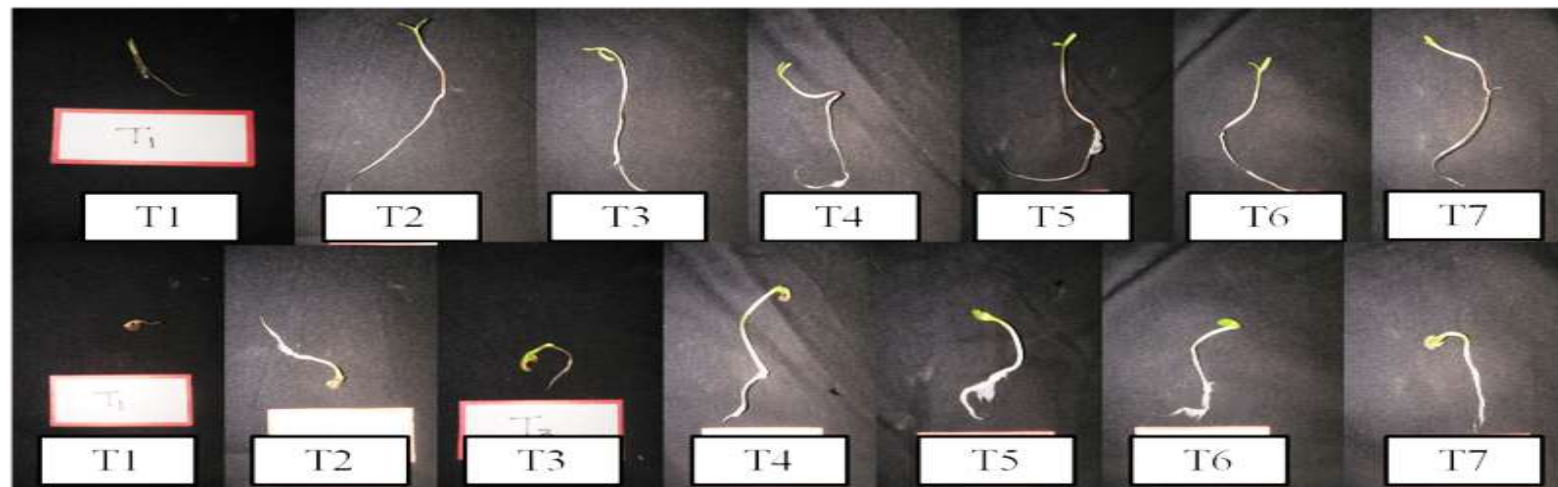


Fig. 4 (a, b) Seedlings of marigold and calendula at 3rd day after treatment

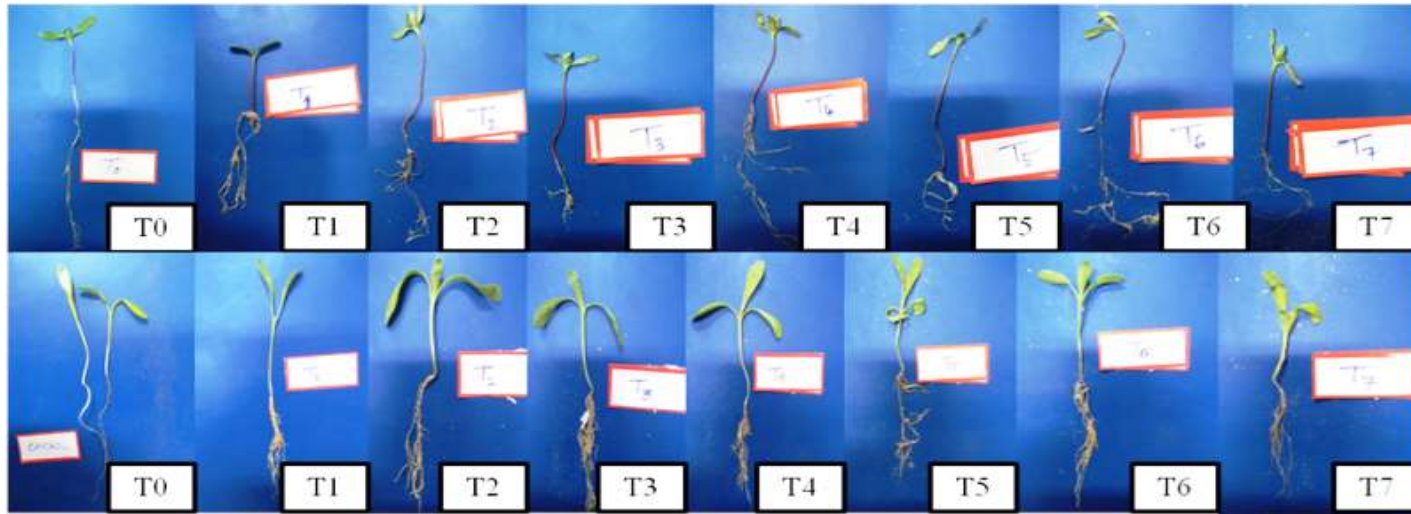


Fig.5 (c, d) 7th day healthy seedlings of marigold and calendula with comparative study