Journal of Asian Scientific Research
ISSN(e): 2223-1331
ISSN(p): 2226-5724
DOI: 10.55493/5003.v12i2.4528
Vol. 12, No. 2, 104-113.

URL: www.aessweb.com

THE INFLUENCE OF DIFFERENT CONCENTRATIONS OF PLANT HORMONES *IN VITRO* ON SEEDS GERMINATION OF FENUGREEK (*TRIGONELLA FOENUM-GRAECUM*)

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Article History
Received: 30 March 2022
Revised: 2 June 2022
Accepted: 17 June 2022
Published: 28 June 2022

Keywords
Trigonella foenum-graecum
Fenugreek
Pharmacological
Seeds germination
Plant tissue culture
Plant hormones
BAP
2,4-D
NAA.

ABSTRACT

Trigonella foenum-graecum is an important leguminous plant used in medicine and human health since ancient times, and the various civilizations of the world, as the Egyptian, Greek, and Indian civilizations, have been used in traditional medicine. *Trigonella foenum-graecum* contains a wide range of pharmacological effects because of the presence of several beneficial plant secondary metabolites. However, the resistant nature of this plant causes major challenges in *in vitro* multiplication, emphasizing the need to assess novel approaches for the propagation of this plant. As a result, this study reports on the effects of BAP, NAA, and 2,4-D on *T. foenum graecum* seed germination *in vitro*. The germination percentage recorded every week for 4 weeks, the parameter's last mean was calculated. The results showed that 2.0 mg/L BAP created the best effect in the parameter studied. The results revealed that 2.0 mg/l BAP provided the greatest response germination percentage (86 ±2.357%). These results highlight the optimum value of growth hormones producing *T. foenum graecum* plant with a minimum germination rate in a large scale.

Contribution/ Originality: This study contributes to the existing literature in plant tissue culture techniques and determines various concentrations of hormones, where this study has been done by using two groups of hormones auxin and cytokinins. The seeds of fenugreek were cultured in media supplemented with BAP, NAA, and 2,4-D hormones.

1. INTRODUCTION

Fenugreek *Trigonella foenum-graecum L.* is an annual leguminous plant from India and the Eastern Mediterranean. Were belongs to the family of Fabaceae, as shown in Table 1 [1-3].

Trigonella foenum-graecum completes the life cycle within a year and usually grows erect, depending on the variety, at the height of around 30-60 cm. The leaves of fenugreek are trifoliate and lobed, while the roots have nodules [4]. Fenugreek flowers are white to yellow, pollinated by insects. It blooms in the middle of summer and has 3-15 cm long skinny hoovesith brownish-yellow seeds flattened by a special type of rhomboid Figure 1 [5, 6]. Fenugreek,

a cultivated medicinal herb found in ancient writings, has antioxidants in its seeds and leaves. From the state of Punjab in India, carbonised fenugreek seeds have been retrieved.

Table 1. Taxonomic Classification of *Trigonella foenum-graecum* [2].

Level of classification	Name
Kingdom	Plantae
Superdivision	Angiosperms
Division	Eudicots
Class	Rosids
Order	Fabales
Family	Fabaceae
Subfamily	Faboideae
Tribe	Trifolieae
Genus	Trigonella
Species	*foenum*

Fenugreek was of commercial importance from 2000 -to 1700 BC India is a significant producer of fenugreek, which is grown worldwide. The crop is harvested around 45,000-55,000 metric tonnes per year. Fenugreek originally came from the "Old World" Mediterranean region or portions of Asia. However, some have theorised that Turkey is its true origin [7-9].

Figure 1. *Trigonella foenum-graecum* Plant [10].

Historically, fenugreek leaves and seeds were used for several therapeutic purposes, including treating mouth ulcers and chapped lips, treating baldness, and alleviating abdominal and abscess pain. These were just a few of the applications of fenugreek in the subcontinent of India, Greece, Arab nations, and China [11-13]. Various genotypes

of fenugreeks are found globally as shown in Table 2, which differ in growth, morphology, seed quality, and crop production. As fenugreek is a self-pollinating plant, it has been effectively produced via breeding methods [14, 15]. *Trigonella foenum-graecum* has been used as a medicinal herb for a long time for many benefits known in traditional medicine. As a result, many studies have been conducted to clarify and prove the medicinal benefits of fenugreek [16], [17]. Seeds of fenugreek containing 25,5% protein, saponins 4.8 %, 20% mucous membranes substances, and 7,9% fat, young leaves and sprouts are a rich protein, mineral and vitamin C source. Polysaccharides, galactomannans, different saponins, such as diosgenins, jamogenins and mucus, are separated from fenugreek seeds, volatile oils, and alkaloids such as choline and trigonelline, Trigonellin, coumarin and niacin were extracted from fenugreek seeds and were helpful for diabetes [18, 19].

Table 2. List of *Trigonella foenum-graecum* species internationally accessible [15].

Fenugreek variety	Country
UM-17, UM-18, UM26, UM-33, UM-50, UM-58, UM-70, UN-79, UM-84, CVT UM-354, CVT UM TC 2336, T-8, HM-46, 1C-74	India
'Ghabrin-6'	Egypt
'Ali Lunghe', 'Ali Corte'	Italy
Ionia	Greece
Gouta	France
Fluorescent, Ethiopian, Barbara, Paul, Kenyan, Moroccan.	England

Fenugreek seeds and leaves also treat high cholesterol and high blood pressure [20]. The leaves contain seven saponin compounds known as graecunins, diosgenin glycosides. Mineral and vitamin content is high in leaves, including phosphorus, iron, carotene, calcium, riboflavin, thiamine, and vitamin C [21], since reduced the levels of total cholesterol in the blood of rats [22]. Fenugreek had a role in remodelling the heart that caused chronic renal failure in the heart of rats caused by adenine and increased the level of antioxidants [23]. Increasing demand for agricultural commodities led scientists to discover techniques to enhance production with excellent nutritional value by utilising contemporary technology; Plant tissue culture is one of these techniques presented by scientists as a solution to the growing demand for agricultural commodities [24, 25]. Plant tissue culture requires an appropriate supply of nutrients, a medium pH, a sufficient temperature, and a sufficient gaseous and liquid or hard environment [26, 27]. This plant tissue culture strategy protects vulnerable species and is the most successful way for increasing crop yield and quality. High-quality plants are produced by separating the genetic elements required to produce genotypes with high yields from adaptable and disease-resistant plants and plants [28, 29]. Where the medium contains essential components such as macronutrients, micronutrients, amino acids or other nitrogen sources, vitamins, organic supplements, carbon sources, solidifying agents, and growth regulators, this composition of the basal medium has a significant impact on plant tissue development and output [30, 31]. Growth regulators are critical for increasing seed germination rates. Externally applied plant growth substances influence a variety of physiological pathways as well as morphogenesis such as germination regulation, stimulation or inhibition of shoot elongation, callus induction, induction of flowering and fruit formation, reduction or increase of fruit set, and speeding or delaying of aging processes such as fruit ripening and defoliation [32].

In a seed, the embryo is the essential structural component. The embryo or small plant is made up of one or more cotyledons, a plumule, a hypocotyl, and a radicle. Many physiological processes are involved in the embryo's growth and development into a new seedling that can stand independently. Breathing, taking in water, enzymatic activation, converting foods into soluble forms for transport, transporting nutrients, water, salts, hormones, and minerals to meristematic areas, and converting foods into plant tissues are all examples of these activities [33, 34]. *In vitro* planting is the process of germinating a seed embryo in a laboratory under favourable growth circumstances similar to that of a biological organism. Seed germination performance *in vitro* is affected by a variety of factors, including culture conditions, seed maturity, carbohydrates, plant growth regulators, and organic additions [35, 36]. *T. foenum-*

graecum includes several key phytochemicals that give birth to numerous therapeutic characteristics required to treat a variety of human health conditions. *T. foenum-graecum in vitro* investigations for phytochemicals and therapeutic properties have expanded dramatically. However, due to the plant's recalcitrance, the application of tissue culture techniques on this plant has been delayed. There is still a gap in *T. foenum-graecum in vitro* seed germination and callus induction utilizing varied hormone doses [4, 37]. This research will identify the best hormone for *T. foenum-graecum* seeds germination and callus induction using plant tissue culture technology. This research will give a dependable way for obtaining sterile *T. foenum-graecum* plant sources. In this study, fenugreek seeds were used as explant for germination by plant tissue culture technique, whereas the seeds of fenugreek sown in Ms media supplemented with different concentrations of auxins Naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins 6-Benzylaminopurine BAP singly and combined.

2. METHODOLOGY

2.1. Materials

2.1.1. Raw Materials

Seeds of *Trigonella foenum-graecum* were obtained from Mercearia Neighbourhood Grocer in Shah Alam, Selangor, Malaysia.

2.1.2. Chemicals

The chemicals employed in the tests include NaCOI, ethanol, DW, MS media, naphthalene acetic acid (NAA), 2,4 Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP), sucrose, and Gelrite agar.

2.2. In vitro Seeds Germination

2.2.1. Sterilisation and Explant Preparation

Soak the fenugreek seeds bought before the night of culture, and then thoroughly wash the seeds with running water for five minutes to remove dust, and contaminants from the surface of the seeds. The fenugreek seeds were then sterilised with 5 percent sodium hypochlorite (v/v) for 20 seconds before being rinsed with sterile distilled water to eliminate the sodium hypochlorite effect. After swirling the seeds in 70% ethanol (v/v) for three minutes. To eliminate the harmful impact of ethanol, seeds were washed with sterile DW once more. To dispose of excess water, the aseptic seeds were placed in a Petri dish on sterile Whatman's filter paper.

2.2.2. Media Preparation and Seeds Culture

MS medium was fortified with various doses of NAA, 2,4-D (0.5, 1.0, and 2.0 mg/L), and BAP hormones (1.0, 2.0, and 3.0 mg/L), both individually and in combination, as indicated in Table 3. In the control group, no hormones were utilised. The medium was hardened by 4 g/L agar before autoclaving, and the pH was setted to 5.8. The media autoclaved at 121°C for twenty minutes. Following autoclaving, 20 ml of media was placed into pillboxes, to cool and solidify before labeling. Using sterile forceps, the aseptic seeds were placed to the hardened MS media. Cultured seeds were preserved for 30 days in a plant tissue culture culture chamber at 25±2 ºC, with 16 hours of light and 8 hours of darkness and relative humidity varying from 40–60 percent. Each treatment comprised 15 pillbox replicas, each with three seeds inserted. Based on the results, the ideal hormone amount for seed germination was determined based on the % of seed germination during this period.

2.2.3. Determination of Seeds Germination

The number of seed germinations was counted after 30 days of culture. Using the equation, the following formula was used to compute the percentage of seed germination for each hormone concentration:

$$\% \text{ seed germination} = \frac{\text{No. of germinated seeds}}{\text{Total number of seeds}} \times 100$$

The final mean value of all parameters was used to calculate the optimal hormone concentration for in vitro seed germination.

2.3. Statistical Analysis

For descriptive statistical analysis, SPSS (Version 26. O) software was utilised, and data were analysed using one-way ANOVA. Tukey's test was employed to differentiate the means.

Table 3. BAP, NAA, and 2,4-D Concentrations for *Trigonella foenum-graecum* Seed Germination *in Vitro.*

Treatment	BAP (mg/L)	NAA (mg/L)	2,4-D (mg/L)
MSO (control)	0	0	0
T1	1	0	0
T2	1	0.5	0
T3	1	1	0
T4	1	2	0
T5	1	0	0.5
T6	1	0	1
T7	1	0	2
T8	2	0	0
T9	2	0.5	0
T10	2	1	0
T11	2	2	0
T12	2	0	0.5
T13	2	0	1
T14	2	0	2
T15	4	0	0
T16	4	0.5	0
T17	4	1	0
T18	4	2	0
T19	4	0	0.5
T20	4	0	1
T21	4	0	2
T22	0	0.5	0
T23	0	1	0
T24	0	2	0
T25	0	0	0.5
T26	0	0	1
T27	0	0	2

3. RESULTS AND DISCUSSION

T. foenum-graecum seeds were germinated in MS medium supplemented with varying doses of the hormones BAP, NAA, and 2,4-D. During imbibition, the seeds absorbed water from the medium, causing the seed coat to swell and soften. The cells started to elongate, and it was observed that all the tip of the radicles had grown out of the seed coat during 15 days of the culture. The process of seed germination had occurred [38].

3.1. Effect of Different Concentrations of BAP on Seeds Germination

Seeds germinated only in one treatment of three different concentrations of BAP hormone tested. The control (MSO) resulted in a negative outcome, with no seed germination. The maximum germination rate of *T. foenum-graecum* was obtained at 2.0 mg/l of BAP, whereas no germination occurred at the other concentrations of BAP over the 30 days of culture. BAP doses of 1.0 mg/l and 4.0 mg/l had no influence on the germination *T. foenum-graecum*

seeds, whereas 2.0 mg/l was recorded (86 ±2.357 %), as shown in Figure 2. Statistical analysis revealed a significant difference (P≤0.05) in seed germination between treatments.

3.2. Effect of Different Concentrations of NAA on Seeds Germination

As shown in Figure 1, *T. foenum-graecum* seeds grew at two NAA hormone concentrations: 0.5 mg/l and 1.0 mg/l; no germination was seen in the 2.0 mg/l concentration after 30 days of culture. The effectiveness of the NAA hormone for stimulating the germination of fenugreek seeds was at the concentration of 0.5 mg/l where seeds germination percentage was 24±0.54%, followed by 1 mg/l NAA hormone with 6±0.54%.

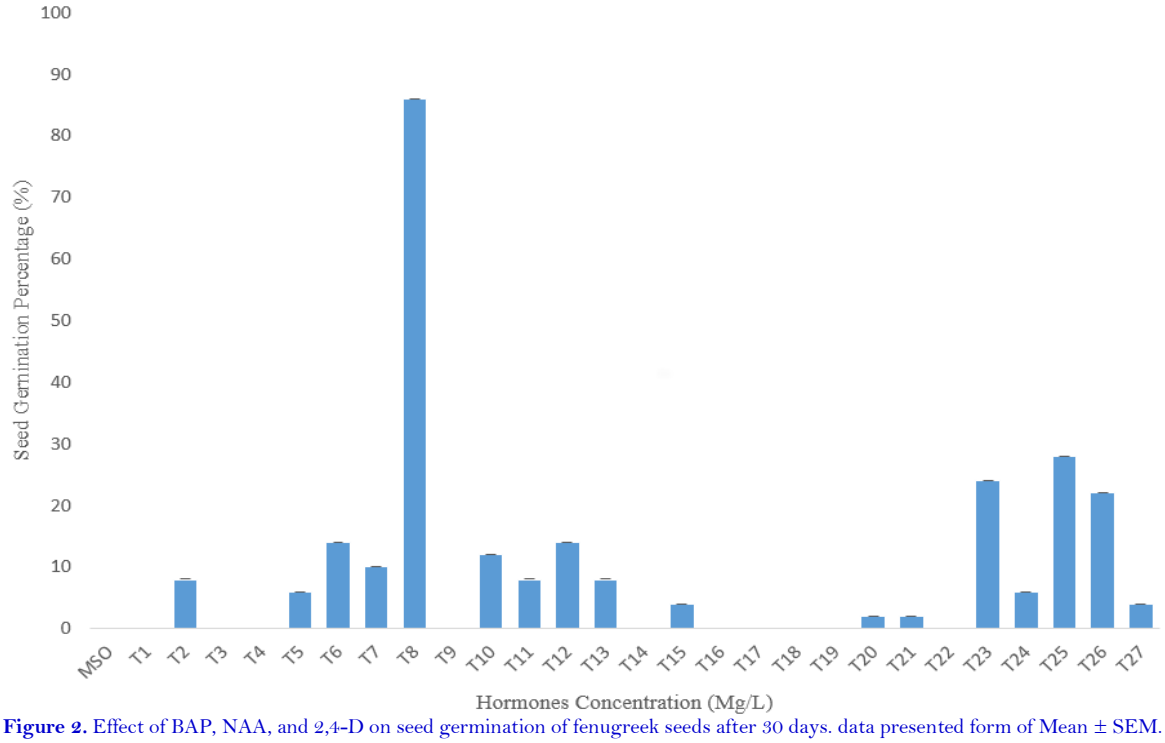

Figure 2. Effect of BAP, NAA, and 2,4-D on seed germination of fenugreek seeds after 30 days. data presented form of Mean ± SEM.

3.3. Effect of Different Concentrations of 2,4-D on Seeds Germination

Seeds germinated in three different concentrations of 2,4-D hormone 0.5 mg/l, 1.0 mg/l and 2.0 mg/l tested. The control MSO has shown a negative response compared with the 2,4-D hormone. The highest percentage of seeds germination was found at 0.5 mg/l followed by 1.0 mg/l of a 2,4-D hormone, while the lowest percentage of seeds germination showed in 2 mg/l of the 2,4-D hormone. The best percentage of germination was at 0.5 mg/l 2,4-D (28 ±1.30 followed by 1.0 mg/l 2,4D (22 ±1.09%). In this study, statistical analysis revealed a significant difference (P≤0.05) between the treatments. The least seed germination percentage appeared in 2.0 mg/l 2,4-D hormone (4 ±0.54%). The percentage of germination began to decrease as the concentration of 2,4-D hormone increased shown in Figure 1.

3.4. Effect of Different Concentrations of BAP Combine with NAA on Seeds Germination

As shown in Figure 1, the combination of BAP and NAA effect the seeds germination occurs in 3 out of the 9 combination concentrations tested. The combination of the BAP with NAA showed the best result for germination when the BAP hormone was at a concentration 2.0 mg/l, and the NAA concentration was 1.0 mg/l. followed by 2.0 mg/l BAP with 2.0 mg/l NAA. Also observed was the presence of germination at a concentration 1.0 mg/l of BAP with a low concentration of NAA 0.5 mg/l. The seed germination percentage was (12±0.83%) for the combination of 2.0 mg/l of BAP with 1.0 mg/l of NAA. While the germination percentage was (8±0.83%) for both treatments, BAP

2 mg/l with NAA 2 mg/l and BAP 1 mg/l combined with 0.5 mg/l. This result observed that the germination rate of *T. foenum-graecum* seeds increased for BAP hormone at concentration 1 mg/l with NAA 0.5 mg/l. The germination rate was zero when used 1mg/l of BAP only without combination in the previous treatment. At the same time, the effect of 2mg/l BAP hormone decreased when combined with any concentration of NAA hormone. Since the effect of 2 mg/l of BAP was very high when used alone in the previous treatment. Where the results were compared directly with the results reported in the previous study demonstrated that the best effect for BAP+NAA hormone was in 1 mg/l BAP+0.5 mg/l NAA and 2 mg/l BAP+2m/l NAA 2 where the germination percentage was 2.66% when the seeds of *T. foenum-graecum* cultured in MS media contain those concentrations of BAP+NAA for 30 days. And the other concentration of BAP+NAA showed no germination at all. Combining BAP hormone with NAA hormone leads to a decrease in the germination percentage of the seeds of *T. foenum-graecum* [4].

Table 4. The influence of various concentrations of BAP, NAA, and 2,4-D on seed germination % of fenugreek seeds after 30 Days. Data presented the form of mean ± SEM.

Hormone Concentration MG/L				
Treatments	**BAP**	**NAA**	**2,4-D**	**Seed Germination % (Mean ± SEM)**
MSO	0	0	0	0 ±0
T1	1	0	0	0 ±0
T2	1	0.5	0	8 ±0.447^{def}
T3	1	1	0	0 ±0
T4	1	2	0	0 ±0
T5	1	0	0.5	6 ±0.547^{def}
T6	1	0	1	14 ±0.895^{cde}
T7	1	0	2	10 ±0.707^{def}
T8	2	0	0	86 ±2.357^{a}
T9	2	0.5	0	0 ±0
T10	2	1	0	12 ±0.836^{cd}
T11	2	2	0	8 ±0.836^{d}
T12	2	0	0.5	14 ±0.574^{cde}
T13	2	0	1	8 ±0.447^{def}
T14	2	0	2	0 ±0
T15	4	0	0	4 ±0.574^{def}
T16	4	0.5	0	0 ±0
T17	4	1	0	0 ±0
T18	4	2	0	0 ±0
T19	4	0	0.5	0 ±0
T20	4	0	1	2 ±0.447^{f}
T21	4	0	2	2 ±0.447^{f}
T22	0	0.5	0	0 ±0
T23	0	1	0	24 ±0.548^{bc}
T24	0	2	0	6 ±0.548^{def}
T25	0	0	0.5	28 ±1.303^{b}
T26	0	0	1	22 ±1.095^{cd}
T27	0	0	2	4 ±0.547^{def}

Note: a, b, c, d, e and f show vertically mean there is a significant difference at $p<0.05$ significant level.

As shown in Figure 1, the combination of BAP and NAA effect the seeds germination occurs in 3 out of the 9 combination concentrations tested. The best result for seeds germination was obtained from 2.0 mg/l BAP + 1.0 mg/l NAA (12±0.83%). This followed by 2.0 mg/l BAP + 2.0 mg/l NAA. The seed germination was also observed at concentration 1.0 mg/l of BAP with a low concentration of NAA 0.5 mg/l. The germination percentage was 8±0.83% for both treatments, BAP 2 mg/l + 2 mg/l NAA, and BAP 1 mg/l + 0.5 mg/l NAA. This result indicates that the germination rate in the *T. foenum-graecum* seeds increased with the BAP hormone at concentration 1 mg/l with NAA 0.5 mg/l where the germination rate was zero when used 1mg/l of BAP alone without any combination. At the same time, the germination percentage decreased when 2mg/l BAP hormone combined with any concentration of NAA hormone. In contrast, the germination rate was very high when the 2 mg/l of BAP was used alone. When the results were compared directly with the results reported in the previous study, the best effect for BAP+NAA hormone was in 1 mg/l BAP+0.5 mg/l NAA and 2 mg/l BAP+2m/l NAA 2 where the germination percentage was

2.66% when the seeds of *T. foenum-graecum* cultured in MS media contain those concentrations of BAP+NAA for 30 days. Besides, other concentrations of BAP+NAA showed no germination at all. Combining BAP hormone with NAA hormone leads to a decrease in the germination percentage of the seeds of *T. foenum-graecum* [4].

3.5. Effect of Different Concentrations of BAP Combine with 2,4-D on Seeds Germination

The combination of different concentrations of BAP+2,4-D hormone leads to germination at different rates. The best results for seeds germination appeared in concentration 1.0 mg/l BAP+1 mg/l 2,4-D (14±0.895%) and 2 mg/l BAP+0.5 mg/l 2,4-D (14±0.574%). The seed germination percentage for concentrations 2 mg/l BAP+2 mg/l 2,4-D, 2.0 mg/l BAP+1 mg/l 2,4-D, and 1 mg/l BAP+0.5 mg/l 2,4-D was 10±0.7.0%, 8±0.447% and 6±0.547%, respectively. The lowest seed germination percentage was found at 4 mg/l BAP+1 mg/l 2,4-D and 4 mg/l BAP+2 mg/l 2,4-D which gave 2±0.447% as shown in Figure 1. Overall, this seed germination study showed that the best hormone for fenugreek was obtained from BAP hormone alone at the concentration of 2 mg/l since it gave the highest percentage of germination, 86 ±2.357% after 30 days of culture. Table 4 shows the influence of various concentrations of BAP, NAA, and 2,4-D on seed germination % of fenugreek seeds after 30 Days. Data presented the form of mean ± SEM.

4. CONCLUSION AND RECOMMENDATION

The current study examined the impact of BAP, NAA, and 2,4-D on fenugreek seeds germination and growth in vitro by analyzing germination percentages grown in different concentrations of BAP, NAA, and 2,4-D. The study showed the ideal hormone for seed germination after 30 days was BAP 2.0 mg/l (86±0.547%), followed by hormone 0.5 mg/l 2,4-D (28±1.303%). It is suggested that future in vitro research be expanded to include various types of hormones on seed germination and utilise varying amounts of those hormones.

Funding: This research is supported by Universiti Teknologi Mara, Malaysia (Grant numbers: MOHE-(600-RMI/FRGS 5/3(49/2015)) and UiTM Grant 600-IRMI 5/3/GIP (024/2018)).
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: All authors contributed equally to the conception and design of the study.
Acknowledgement: The authors thank the Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam Branch for the technical support towards this research.

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