Int.J.Curr.Microbiol.App.Sci (2019) *8*(3): 866-876

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 03 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.803.104

Effect of Phytohormones and Other Dormancy Breaking Chemicals on Seed Germination of *Inula racemosa* Hook F. – A Critically Endangered Medicinal Plant of North Western Himalaya

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Keywords

Seed germination, Chilling, Hormone, Endangered, *Inula racemosa*

Article Info

Accepted:
10 February 2019
Available Online:
10 March 2019

A B S T R A C T

Inula racemosa Hook. f. is commonly called Pushkarmool, belongs to family Asteraceae. The plant is distributed in temperate alpine Himalayas at an altitude of 1,500- 4,200m from Kashmir to Kumaon, Afghanistan to Central Nepal. Pushkarmool is commercially a very important medicinal plant of the North Western Himalayas. The plant is used in Ayurveda as an expectorant, rejuvenator and immune modulator. Root powder is reportedly hypoglycemic and hypocholesterolemic in human subjects. The species is critically endangered because of the fragile nature of its habitat, habitat destruction, illegal harvesting from the wild source, high market demand and destructive harvesting practices. The objective of the present study was to develop effective pre-sowing treatments to improve the seed germination, and to reduce mean germination time, so that protocols for commercial production of the plant could be developed and natural populations restored to ensure proper conservation of the species. In the present study phytohormones and other seed dormancy breaking chemicals were used to investigate their effect on seed germination. The results revealed highest mean percentage of germination in chilling treatment Ch_1 (30 day chilled seeds) followed by treatment $GA_3$1 (Gibbrellic acid, 10^{-3} M) in almost all day intervals, barring 16th day interval where highest mean percentage of germination was observed in chilling treatment Ch_2 (40 day chilled seeds) and least was observed in Com. 1 and control treatments. On 30th day, the highest mean percentage of germination ± SE (82±1.15) was observed in treatment Ch_1 followed by $GA_3$1 (79±1.73) and least (24±1.15) was observed in treatment Com .4 (H_2SO_4/NAA2). However, no germination was observed treatments K_1 (Potassium nitrate, 0.2 %,) and K_2 (Potassium nitrate, 0.3 %). Treatment S_1 (Acid scarification for 1 min.) with mean percentage of germination ± SE (31±1.15) was at par with treatment Com 2 (H_2SO_4/$GA_3$2), but was statistically different from all other treatments. All the treatments were statistically significant. On 28th day, the highest mean percentage of germination ± SE (82±1.15) was observed in treatment Ch_1 followed by Ch_2 (78±1.15) and $GA_3$1 (74±1.15) least (23±1.15) was observed in treatment Com .4. From day 14th to day 26th, treatments $GA_3$1 and $GA_3$2 (Gibbrellic acid, 10^{-4} M) were statistically different from each other as well as from the rest of all other treatments. From day 6th to day 18th, treatments NAA1 (Naphthalene acetic acid, 25 ppm) and NAA2 (Naphthalene acetic acid, 50 ppm) were at par with each other, but from day interval of 20th to 26th these treatments were statistically different from each other. Treatments were statistically significant on all day intervals. The germination study gives an insight into the measures to be taken to increase the efficiency of seed germination to ensure the conservation of the endangered species.

Introduction

Germination is a critical stage in the life cycle of every crop plant and often controls population dynamics, with major practical implications. Seed germination is the critical stage for species survival (Huang *et al.,* 2003 and Yang *et al.,* 2008). Seed germination studies proved to be useful in development of conservation study (Kandari *et al.,* 2007). Seeds may be non-dormant at maturity and thus germinate soon after dispersal if environmental conditions are favourable for them germinates. However, favourable conditions may not persist long enough for the resulting plant to become established. Seed dormancy prevents seeds from germinating under unfavorable conditions, thus reducing the chances of seedling mortality and there by contributing to the success of population regeneration (Gutterman, 2012). However, poor seed germination of viable seeds in several Himalayan plant species is experienced as a limiting factor in multiplication of plants at a large scale (Baskin and Baskin, 2014 and Nadeem *et al.,* 2002).

Inula racemosa is commonly called Pushkarmool belongs to family Asteraceae. The plant is distributed in temperate alpine Himalayas at an altitude of 1,500- 4,200m from Kashmir to Kumaon, Afghanistan to Central Nepal (Firdous, *et al.,* 2018). Pushkarmool is a very important medicinal plant of the North Western Himalayas (Anonymous, 1998 and Wani *et al.,* 2006). The plant is used in Ayurveda as an expectorant and resolvent in indurations. It is considered as a 'Rasayana'(rejuvenator, immunomodulator) by Ayurvedic physicians. According to Bhavaprakasha the drug is bitter pungent in taste (Bhavaprakasha, 1961). The aqueous extract of the fresh or dry roots is given orally in rheumatic pains and liver problems. Externally a paste or liniment is used for relieving pain. The root is also used in veterinary medicine as a tonic. The root forms an important ingredient of several polyherbal formulations for heart diseases and inflammatory conditions of spleen and liver. Along with *Commiphora mukul*, the drug combination called 'Pushkarguggulu' is a popular anti obesity, hypolipidemic indicated in cardiac ailments (Firdous *et al.,* 2018). In Chinese medicine it is used for abdominal distension and pain, acute enteritis and bacillary dysentery (Pharmacopoeia Committee of the Ministry of Health 1995). Root powder is reportedly hypoglycemic and hypocholesterolemic in human subjects (Tripathy *et al.,* 1979). It brought about a beneficial improvement in ECG of patients with Ischemic heart disease (IHD) (Tripathy *et al.,* 1983). At 450 mg/kg it enhanced PGE2 like activity in isoproterenol induced myocardial ischemia in rabbits.

In combination with guggulu it is anti anginal and hypolipidemic in patients with IHD (Ramji *et al.,* 1991). In multi herb combination with *Terminalia arjuna* and *Commiphora mukul*, it is reported to exert cardioprotective effect in isoproterenol induced myocardial ischemia in rats (Seth *et al.,* 1998). Zhang *et al.,* (2012) isolated sesquiterpenes from the roots of *Inula racemosa* Hook. f. and later they evaluated these compounds for their inhibition of LPS-induced nitric oxide production in RAW264.7 macrophages, and the results indicated that some compounds moderately inhibited nitric oxide production with IC50 values of 7.39 ± 0.36, 6.35 ± 0.26, and 5.39 ± 0.18 μM, respectively. He *et al.,* (2014) reported that the ethanol extract of *I. racemosa* roots and its constituent compounds have potential for use in the control of *A. albopictus* larvae.

The populations of the species in the entire North Western Himalayan range are

witnessing a speedy decline in density, dwindling both in size and number (Shabir *et al.,* 2010*).* The populations of the species in the entire north western Himalayan range are witnessing a speedy decline in density, dwindling both in size and number (Parvaiz *et al.,* 2006).

As the species is a critically endangered because of the fragile nature of its habitat, habitat destruction, illegal harvesting from the wild source, great market demand and destructive harvesting practices, the objective of the present study was to develop effective pre-sowing treatments, to improve the seed germination, so that protocols for commercial production of the plant could developed and natural populations could be restored to ensure proper conservation of the species.

Materials and Methods

Seeds of *Inula racemosa* were collected at maturity from alpine natural populations from Mahadave hills of Kashmir Himalaya (3000–4000 m asl) during August to September 20017. The seeds were air-dried for a fortnight at room temperature (15±2°C) and then were stored at room temperature (15±2°C). Seeds were washed with 0.1% mercuric chloride for 5-7 minutes and then with 70% alcohol for 1 minute and thoroughly rinsed with double distilled water and divided into groups of 50 seeds each.

Physical treatment

Stratification/Chilling

The surface sterilized seeds (using mercuric chloride) were soaked in distilled water for 24 hours and then subjected to chilling at low temperature (3-4ºC) for different durations [(Chilling treatment (Ch): Ch1 = 30 days, Ch2 = 40 days and Ch3 = 50 days)] using Refrigerator (Make L.G.).

Acid scarification/Sulphuric acid (H2SO4) treatment

The seeds were treated with concentrated sulphuric acid for 1 min: S1 followed by thorough washing in distilled water. Sulphuric acid (Sigma) used was 99.9% pure.

Chemical treatment

Potassium nitrate (KNO_3)

Surface-sterilized seeds were moistened with different concentrations of aqueous solution potassium nitrate (K1 = 0.2%, K2= 0.3%) for 24 hours followed by germination on substratum moistened with different concentrations of aqueous solution potassium nitrate (K1= 0.2%, K2= 0.3%).

Gibbrellic acid and naphthalene acetic acid treatment

The surface sterilized seeds shall be kept submerged in aqueous solution of GA_3 and NAA with a concentration of 10^{-3} M and 10^{-4} M ($GA_3$1= 10^{-3} M, $GA_3$2= 10^{-4} M, NAA1=25 ppm, NAA2= 50 ppm) for 24 hours.

Combined treatment (Combination of sulphuric acid and hormones)

Seeds were treated with H_2SO_4 for 1 min followed by through washing and then soaking in solution of hormone (GA_3 solution and NAA solution) for 24 hours and subsequent germination on substratum moistened with GA_3 s and NAA solution {(Combined treatment (Com) H_2SO_4/GA_3: Com 1= $H_2SO_4/GA_3$1, Com 2= $H_2SO_4/GA_3$2) (Com) H_2SO_4/NAA: Com 3= H_2SO_4/NAA1, Com 4= H_2SO_4/NAA2)

One treatment was kept as control (Cont). There were fifteen treatments; fifty seeds in triplicate were used for each treatment. The

experiment was laid in complete randomized design (CRD) with 3 replications each. After the treatment, the seeds were subjected to germination test by allowing them to germinate on the moistened filter paper. The germination of the seeds was monitored over the next one and a half month at an average temperature of 15-20ºC. Observation on the no. of days taken for the first seed to germinate, total no of days for complete germination and the total no of seeds germinated were noted on regular basis. One way ANOVA was used to find out various statistical terms. The relative effectiveness of different physio-chemical and hormonal treatments in dormancy removal and germination improvement was calculated and the seedlings were transferred to the pots.

Results and Discussion

Seed germination is a complex process, which starts with the absorption of water, followed by a short pause, and ending with the synthesis and activation of enzymes. Germination is regulated through a series of interactions of hormonal and environmental factors, and this is possible only when appropriate conditions are met (Aticia *et al.*, 2003). Germination occurs as a result of the partial exposure of the cotyledons of the seeds which permits the process of hydrolysis whereby hormones, increase nucleic acid metabolism and protein synthesis, are released (Uwaegbute, 1996). In the present study germination started in different treatments on different dates. It started on day 2nd in chilling treatments Ch_1 and Ch_2 and was observed till 30th day in all the treatments (Table 1). The highest mean percentage of germination was observed in chilling treatment Ch_1 (30 day chilled seeds) followed by treatment GA31 in all most all day intervals and least was observed in Com. 1 or control treatments, barring 16th day interval where highest mean percentage of germination was observed in chilling treatment Ch_2.

On 30th day, the highest mean percentage of germination ± SE (82±1.15) was observed in treatment Ch_1 followed by $GA_3$1 (79±1.73) and least (24±1.15) was observed in treatment Com. 4. However, no germination was observed treatments K_1, and K_2 (Table 2 and 3). Chilling treatments Ch_1 and Ch_2 were at par with treatment $GA_3$1 and treatment Ch_3 was at par with $GA_3$2. All the three chilling treatments were statistically different from each other as well as with the rest of other treatments. Treatment S1 with mean percentage of germination ± SE (31±1.15) was at par with treatment Com 2, but was statistically different from all other treatments. Treatment $GA_3$1 was at par with treatment Ch_1 and Ch_2 and $GA_3$2 was at par with treatment Ch_3, but were statistically different from each other and from all other treatments. Treatment NAA1 and NAA2 were statistically different from each other as well as from the rest of other treatments. Treatment Com 1 and Com 2 were statistically different from each other as well as from the rest of other treatments. However, treatment Com 2 was at par with treatment S1. Treatments Com. 3 and Com. 4 were at par with each other, but were statistically different from other treatments. Control treatment was statistically different from other treatments. All the treatments were statistically significant and treatment Ch_1 was the best treatment among all other treatments.

On 28th day, the highest mean percentage of germination ± SE (82±1.15) was observed in treatment Ch_1 followed by Ch_2 (78±1.15) and $GA_3$1 (74±1.15). Least germination (23±1.15) was observed in treatment Com. 4 (Table 2 and 3). Chilling treatments Ch_1 and Ch_2 were at par with each other, treatment Ch_2 was also at par with treatment $GA_3$1 but were statistically different from the rest of other

treatments. Treatment S1 was at par with treatment Com 1, but was statistically different from all other treatments. Treatment $GA_3$1 was at par with treatment Ch_2 and Ch_2 and $GA_3$2 was at par with treatment Ch_3, but were statistically different from each other and from all other treatments. Treatment NAA1 and NAA2 were statistically different from each other as well as from the rest of other treatments. Treatment Com 1 and Com 2 were was at par with treatment S1, however, treatment Com 2 was also at par with treatment Com. 3, but both treatments Com 1 and Com 2 were statistically different from each other as well as from the rest of other treatments. Treatments Com. 3 and Com. 4 were at par with each other, but were statistically different from all other treatments. Control treatment was statistically different from other treatments. All the treatments were statistically significant.

From day 14^{th} to day 26^{th}, treatments $GA_3$1 and $GA_3$2 were statistically different from each other as well as from the rest of all other treatments. From day 6^{th} to day 18^{th}, treatments NAA1 and NAA2 were at par with each other, but from day interval of 20^{th} to 26^{th} these treatments were statistically different from each other. Treatments were statistically significant on all day intervals.

The highest mean percentage of germination in chilling treatment Ch_1 (30 day chilled seeds) in almost all day intervals may be because chilling performs a pivotal role in inducing the stimulus that is needed to surmount dormancy. It is regarded to initiate an increase in the concentration of gibberllic acid (Bretzloff and Pellett, 1979). Chilling is useful to relieve primary inactiveness of many Northern hemisphere species (Baskin, 2001). It has been commonly used as a pre-sowing treatment to overcome dormancy and enhance percentage of germination of dormant seeds of many different species (Fang *et al.,* 2006). The pre-chilling treatment conditions may actually be simulating the events that occur during the winter season just before the appearance of summer.

The next higher mean percentage of germination was observed in treatment GA31 (Fig. 1). It seems that pre chilling requirement in this species is replaced by gibberellin treatment, as it took only 30 days for complete germination. However, seeds were chilled for varying durations (1-month, 40-days and 50 days) before observing their germination over a period of one month. So treatment of seeds with GA3 saves a time period of approximately one month. Similar results have been reported by Sharma *et al.,* (2006) while studying the seed germination behavior of some medicinal plants species. However, our results were contradictory to Shabir *et al.,* (2010) as they did not obtain any germination in gibberellin treated seeds. Gibberellins surmount seed and bud dormancy in many species, thus serving as a substitute for low temperatures, long days or red light (Salisbury and Ross, 1992). In the present study, this response to germination was influenced by proporition of applied GA3. At lower concentrations (GA32= 10-4 M), germination was lower (75%) and at higher concentration (GA31= 10-3 M), it was higher (80%) (Table 1 and Fig. 1). Dormant seeds, which demand cold temperature treatment, dry storage following maturation as initiator or stimulator of germination are mostly treated with GA3 to surmount their dormancy (Nadjafi *et al.,* 2006). Plant growth hormones are chemicals which in small quantities can regulate various plant processes in addition to seed dormancy. Different plant hormones can control different plant processes including seed dormancy and germination, growth and development of various plant parts (Agraeber *et al.,* 2012). Gibberellins are mostly employed to destroy the low temperature requirements of some

plant seeds and enhance their germination percentage (El-Dengawy, 2005). It plays a role in inducing enhancement of enzyme synthesis that changes stored nutrients carbohydrates, which are required for quick cell respiration during germination (Bakrim *et al.,* 2007). Similar mean percentage of germination ± SD (70±2.3) was observed in treatments $GA_3$2 (10-4 M) and Ch_3 (50 day chilled seeds), respectively, again justifying the positive role of gibberellins in enhancement of germination.

Increase in germination per centage was reported by other workers from studies carried out on other species, such as *Ferula gummosa* (Nadjafi *et al.,* 2006), *Sesamum indicum* (Kyauk *et al.,* 1995) and *Rumex dentatus* (Ali *et al.,* 1996).

Absence of germination in treatments K1 and K_2 (KNO3, 0.1% and 0.2) may be because of the fact that application of nitrogen, in KNO3 form cause a toxic effect, thus inhibiting seed germination or this could be attributable to oxygen deficiency due to impermeable testa, or build-up of inhibitors as well as salinity (toxic effect) of the chemical (KNO_3) (Nego, 2015). Similar results have been reported by Fernández *et al.,* (2006) while studying seed germination of *Rumex crispus.* Pérez Fernández and Rodríguez-Echeverría, 2003 on ten species of wood lands and Bhardwaj *et al.,* (2016) in *I. racemosa* also found inhibition in seed germination by exogenous application of KNO_3.

Low percentage of germination in sulphuric acid treated seeds (S_1) (Fig. 1) may be because of the fact that acids have negative influence on seed germination. Conc. Sulphuric acid breaks the phospho-diester bonds, which serve as a backbone for DNA, resulting in damage to DNA and cells. Thus the embryo gets killed. Similar results were observed by Nasiri and Eisavand (2001) and Shabir *et al.,* (2010) while studying the influence of acidic treatments on germination of *Ceratonia siliqua* and *I. racemosa,* respectively. Contrary to the present findings, Saied *et al.,* (2008) on *Ziziphus,* Khaleghi *et al.,* (2009), on Tamarind, Nasiri and Eisavand (2001), on *Albizia julibrissin* and Hojati *et al.,* (2007), on *Cycas revolute* Bhardwaj *et al.,* (2016) on *Inula racemosa* had introduced sulfuric acid as the best treatment.

In naphthalene acid treated treatments NAA1 and NAA2, mean percentage of germination ± SD (66±21.6 and 53±16.77) was higher as compared to control treatment (41±14.19). Shinde *et al.,* (1994) also reported increased percentage germination as well as seedling vigour with the exogenous application of naphthalene acetic acid (NAA). However, it was lower compared to gibberllic acid treated treatments GA31 (79±27.04) and GA32 (70±23.30) treatments. This is because of the positive effects of hormone naphthalene acetamide, (synthetic auxin that acts as a rootining harmone) on the seed coat as the partial exposure of the cotyledons of the seeds permits the process of hydrolysis whereby hormones such as auxins and ethylene, which could increase nucleic acid metabolism and protein synthesis, are released (Uwaegbute, 1996). Maku *et al.,* (2014) also reported higher percentage of germination in *T. tetraptera* seeds when treated with naphthalene acetic acid (NAA).

In case of treatments Com 1(H_2SO_4/$GA_3$1) and Com. 2 (H_2SO_4/$GA_3$1), mean percentage of germination ± SD (37±11.84 and 31±10.34) was lower compared to control treatment (41±14.19). It may be because of the negative effect of sulphuric acid on seed germination**.** Similar was the case with treatments Com 3 (H_2SO_4/NAA1) and Com. 4 (H_2SO_4/NAA2). Our results are contradictory to Bhardwaj *et al., (*2016). They reported higher percentage of germination in acid

treated treatments as compared to control treatment. As treatment of seeds with conc. sulphuric acid may be injurious to the seeds as acid may rapture vital parts of the embryo. According to Levitt (1974) and Nikoleave, (1977) immersion of seed in concentrated sulphuric acid disrupts the seed coat. However, Shabir *et al.*, (2010) reported higher percentage of germination in *I. racemosa* seeds that were scarified and then treated with gibberellin.

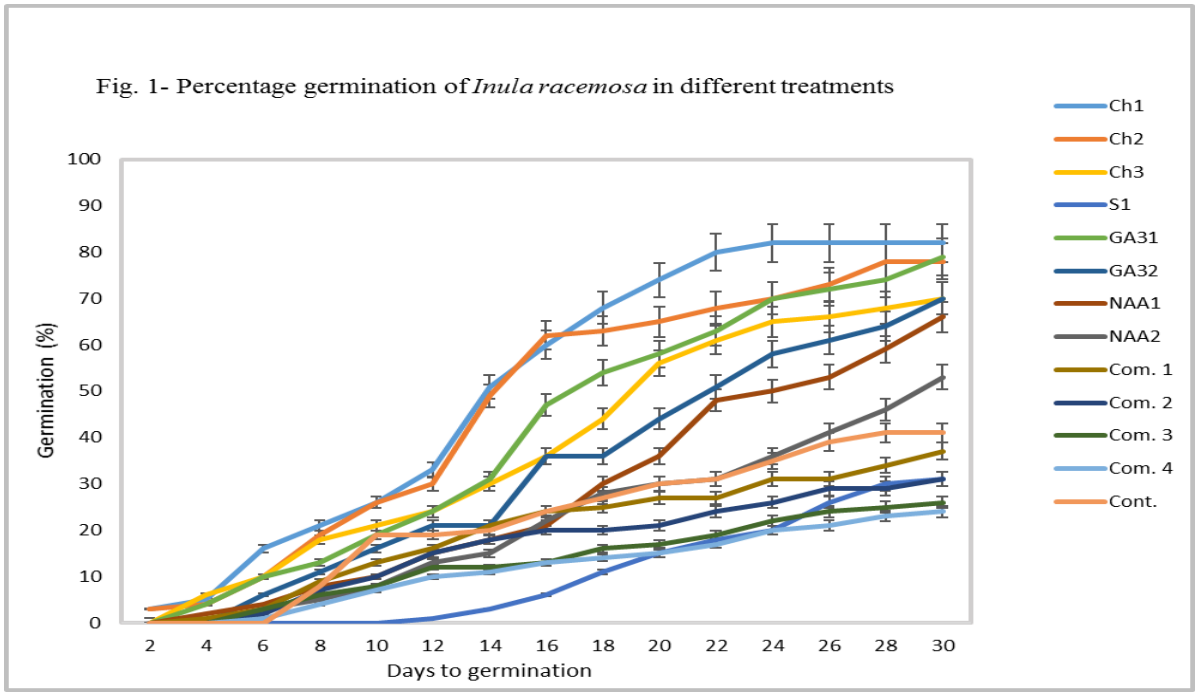

Table.1 Seed germination studies of *Inula racemosa* Hook. F.

Treatments	No. of days taken for Ist seed to germinate	No. of days taken for last seed to germinate	Total no. of days taken for complete germination
Ch_1	1	26	26
Ch_2	1	30	30
Ch_3	3	32	30
S_1	11	28	15
$GA_3$1	4	30	27
$GA_3$2	5	33	29
NAA1	4	30	27
NAA2	6	31	26
Com 1	5	30	26
Com 2	6	30	25
Com 3	6	30	25
Com 4	6	29	24
Cont.	8	39	32

{Chilling treatment (Ch),: Ch1= 30 days, Ch2= 40 days, Ch3= 50 days: Potassium nitrate (K) with K1= 0.2 %, K2= 0.3%: Gibbrellic acid (GA_3) with $GA_3$1= 10^{-3} M and $GA_3$2= 10^{-4} M: Combination of sulphuric acid and GA_3 (Com), Com 1= $H_2SO_4/GA_3$1, Com 2= $H_2SO_4/GA_3$2: Sulphuric acid treatment= S1: Control =Cont.}

Table.2 Statistical parameters of seed germination of *Inula racemosa* during different day intervals

Day intervalls	Critical difference	standard error (d)	Coefficient of variation
Day 2	0.43	0.21	64.55
Day 4	1.06	0.51	43.12
Day 6	2.20	1.07	29.04
Day 8	2.66	1.30	18.50
Day 10	3.20	1.56	15.69
Day 12	3.82	1.86	14.68
Day 14	3.67	1.79	11.00
Day 16	4.93	2.40	11.50
Day 18	4.36	2.12	8.97
Day 20	3.84	1.87	7.05
Day 22	4.81	2.34	8.01
Day 24	4.66	2.27	7.13
Day 26	4.18	2.03	6.02
Day 28	4.03	1.96	5.53
Day 30	3.43	1.67	4.468

Table.3 Mean percentage of germination on 28th and 30th day of seed treatment

Treatment	Mean± S.E.	Mean± S.E.
Ch_1	82.00±1.15	82.00±1.15
Ch_2	78.00±1.15	78.00±1.15
Ch_3	68.00±1.15	70.00±1.15
S_1	30.00±1.15	31.00±1.15
$GA_3$1	74.00±1.15	79.00±1.73
$GA_3$2	64.00±1.15	70.00±1.15
NAA1	59.00±2.30	66.00±1.15
NAA2	46.00±0.57	53.00±1.15
Com 1	34.00±1.155	37.00±0.57
Com 2	29.00±2.30	31.00±1.15
Com 3	25.00±0.57	26.00±0.57
Com 4	23.00±1.73	24.00±1.15
Cont.	41.00±2.30	41.00±2.30
C.D.	4.03	3.43
SE(d)	1.96	1.67
C.V.	5.53	4.46

{Chilling treatment (Ch),: Ch1= 30 days, Ch2= 40 days, Ch3= 50 days: Potassium nitrate (K) with K1= 0.2 %, K2= 0.3%: Gibbrellic acid (GA_3) with $GA_3$1= 10^{-3} M and $GA_3$2= 10^{-4} M: Combination of sulphuric acid and GA_3 (Com), Com 1= H_2SO_4/$GA_3$1, Com 2= H_2SO_4/$GA_3$2: Sulphuric acid treatment= S1: Control =Cont.}

In conclusion, it is recommended that the most practical and useful pre-treatments for propagation of *Inula racemosa* are the pre-chilling treatment for a period of 40 days at low temperature (3-4ºC), and treatment with GA_3. As in *I. racemosa* low temperature requirement is replaced by GA_3. The plant species could be produced in bulk quantity

when the seeds are treated with GA_3. The application of GA_3 has also resulted in the reduction of germination time period from 2-2.5 months to a month.

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How to cite this article:

Haleema Bano, Javeed I.A. Bhat, F.A. Lone, Faisal Noor, M. Ashraf Bhat and Nageena Nazir. 2019. Effect of Phytohormones and Other Dormancy Breaking Chemicals on Seed Germination of *Inula racemosa* Hook F. – A Critically Endangered Medicinal Plant of North Western Himalaya. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 866-876.
doi: https://doi.org/10.20546/ijcmas.2019.803.104