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Standardization of propagation and agro techniques in *Ginkgo biloba* L. - A medicinally important plant

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Abstract
Due to the high medicinal value and high demand of its herbal products globally, it has been exploited indiscriminately and facing high risk of extinction. An efficient and sustainable protocol has been developed for its propagation by semi hard stem cuttings and by seeds also. Some growth hormones have been used in different concentrations. The highest sprouting (%) was recorded in the Catchin acid at 10 mg/l (96.67%) and lowest in Tanic acid 2.5 mg/l (24.0%). The statistically significant rooting (%) and root length/plant in 2nd year was recorded in IBA 250 mg/l (85.33%). In IBA + Phloroglucinol, 250+10 mg/l, and Salicyclic acid 25 mg/l, (7.33) and average root length/plant were recorded in IBA 250 mg/l, (13.67cm). In NAA 250 mg/l (14.67cm). The statistically significant seed germination (%) in IBA 250 mg/l (85.74%) and NAA in 500 mg/l (84.73%) was also comparable. (FYM doses viz, 15, 30, 45 & 60 t/ha with control) were recorded in monthly interval from 2005 to 2013 statistically analyzed on a yearly basis. The purpose of FYM x Spacing trial was to study the potential of leaf production for commercial use. In 2009 to 2013, FYM 30 t/ha produced highest quantity of leaves.

Keywords: *Ginkgo biloba* L., Indole butyric acid, Indole acetic acid, Phenolic acids, FYM, Spacing

1. Introduction
Ginkgo biloba L. mentioned in the Chinese Materia Medica about 5000 years ago by Deng. In the world scenario, *Ginkgo biloba* was admitted as a living fossil of Jurassic period and emphasizes its relic position (Jacobs and Browner, 2000) [13]. *Ginkgo biloba* L. (Ginkgoaceae) is a native plant of China. It was first introduced in Chinese herbals around 14th century A.D. for its fruits "fruit" that was consumed raw or cooked. Its existence was doubtful as wild state, but it may still occur in some of the remote mountain valleys of Zhejiang province (Eastern China) as well as other parts of China. The name of the species, biloba recall due to the bilobed shape of the leaves. The similarity of the leaves has to shape and vein, with those of the maidenhair fern. In China and Japan it is held sacred and is cultivated in temple gardens. It was introduced into Europe around 1730 and is now widely cultivated as an ornamental tree in the streets and parks in western countries and as a medicinal plant, particularly in China, Korea, France, Germany and United States. The male trees are more desirable for planting, because female plants produce many ill smelling seeds. Ginkgo is a tree of great beauty, with a long span, highly resistant to insects, bacterial, viral infections and air pollution. As per the report by a London based management consultancy reports that *G. biloba* is a species in urgent need of sustainable conservation because of its use in herbal medicines (Masood, 1997) [23] globally.
The medicinal properties of *G. biloba* seeds were reported in "Pen Ts'ao Kang MLL" (Great Herbal by Li- Shih chen in 1596). The uses of *G. biloba* for treating allergies, alzheimer's disease, headache, asthma, tinnitus, impotence, circulatory disorders, eye disorders, diabetes, multiple sclerosis, brain-trauma and as a free radical scavenger has been reviewed (Murray 1996) [25]. The biology and chemistry in reference to History, nomenclature and systematic, distribution, Ecology, sexual reproduction, seed dispersal and establishment, tree architecture, seed production, leaf production, cultivation and propagation, pharmacological importance, secondary metabolites and economical importance of the plant has also been reviewed (Singh *et al.*, 2008) [38]. Variations in secondary metabolite were studied by Kaur *et al.*, (2012) [16] and the presence of terpene trilactones (Kaur *et al.*, 2009) [15]. The extract of *G. biloba* leaves have been used in the cardio protective mechanism, myocardial ischemia and reperfusion injury

(Shen and Zhou, 1995) [37]. Some workers have reported its efficiency against environmental pollution (Sharma, 1989; Neinhuis and Barthlott, 1998). Huang *et al.*, (1990) [36, 27, 12] has reported tolerance to SO_2 and accumulation of sulphur (s) in 51 species of broad leaves deciduous trees, evergreen coniferous trees and deciduous shrubs etc. in Beijing. The plant is becoming extinct due to high exploitation by pharmaceutical industries all over the world. Purohit *et al.*, (2009) [32], has reported *G. biloba* Linn a Living Fossil under threat due to illegal exploitation, anthropogenic pressure and lack of knowledge about sustainable harvesting. We have tried to establish it all over India by planting plants raised through vegetative propagation using semi hard stem cuttings and through seeds. The elite plants were selected and propagated further in nursery and transferred in the experimental field. As per the surveys of India (54 number in five years), more than 50% population of *G. biloba* was found near the extinction stage. Because it is a rare species and of high demand globally, about 26 to 32% demand of its extract and product increasing annually. The technique of its vegetative propagation and multiplication is developed through sustainable agro techniques for its conservation to save this species from extinction. The standardized technique of propagation has been applied practically for raising large scale nurseries and commercial plantation in India. Over 30,000 (thirty thousand) nursery plants has been raised in IHBT Palampur (H.P.). More than eight thousand plants have already been distributed all over India for plantation, commercial use and its conservation purpose.

2. Importance of Conservation

As per the report by a London based management consultancy that *G. biloba* is a species in urgent need of conservation because of its use in herbal medicines. The demand of *G. biloba* in the world is increasing from 26 to 32% every year. So, the urgent need to cultivate *G. biloba* at a large scale is highly required (Masood, 1997) [23]. *Ginkgo biloba* is considered the oldest tree species, surviving on earth. It has been recorded after 54 numbers of surveys conducted in five years during 2004 to 2008 all over India. In India about 30 number trees of *G. biloba* are recorded, about 60% are below the age of 30 to 35 years, Rest about 40% are in semi dried condition i.e. just about to die due to illegal commercial exploitation (Fig.1). Most of the branches and bark has been removed from the plant. The author has already reported the current status of *G. biloba* in India (Gopichand *et al.*, 2009) [23]. Charles Darwin called *G. biloba* is a 'living fossil'. In different continents, *Ginkgo* is reported to have achieved climax of its diversity during the Jurassic period, which declined rapidly at the end of the early Cretaceous period (Parkash and Kumar, 2004) [31]. Fossil of this species in the Mesozoic era have been discovered in its native rouge (Eastern China). It's all relatives became extinct in other parts of the world but *G. biloba* was only species which survived, even after in the world war 1st and 2nd. It was introduced in Europe from Japan in 1730 and in the USA 1784 as an ornamental tree (http://w.w.w.worldagroforestrycentre.org. The leaves of *ginkgo*, documented in Chinese medicine as bai-guo-ye as first mentioned in Lan Mao's Dian Nan Ben Cao (Pharmaceutical Natural History of Southern Yunnan). It was published in 1436 during the Ming dynasty, commercial work recorded in 1505 (Del Tredici, 1991) [6]. More than 300 scientific studies have been conducted in chemistry, pharmacology and clinical effects of its leaf. On the basis of experimental studies, it has a new era in human society in a wide variety of clinical conditions, vertigo, and tinnitus, treatment of poor circulation, heart disease, eye diseases, chronic cerebral insufficiency, brain trauma, dementia, and senility, bacterial and fungal infections. New uses for ginkgo leaf extracts emerging are used in circulatory problems, erectile dysfunction, memory, blood circulation. The Egb 761 has more beneficial properties, leaf bioflavanoids (ginkgolides A,B,C,J bilobilides). PAF is used in inflammatory, cardiovascular, respiratory disorder etc. It is used against pollution, especially in urban forestry, nitrogen fixing, root of *Ginkgo biloba* colonized by the fungus *Glomus epigaeum*, forming vesicular arbiscular mycorrhiza.

From above quoted good features it is urgently required to conserve and establish its plantation as many as possible. The Indian Government has also supported the aim and objective of this programme and sanctioned two projects, phase-I and phase-II from National Medicinal plant Board Govt. of India, New Delhi.

3. Materials and Methods

The experimental work was conducted during 2005 to 2013 in Biodiversity farm of CSIR-IHBT Palampur (1325m, msl, 32^0 06'05"N $76^0$34'110"E) situated in the mid hill in Himachal Pradesh India. The semi hard stem cutting was collected from over own field. The meteorological data during the study period has been recorded by CSKHPKV, Palampur (Table 1).

Table:-1 The weather data (averages in yrs) of experimental site in Palampur (H.P.)

Years	Temperature °C		Relative Humidity (%)		Bright Sun	Rainfall	Evaporation
	Max	Min	RH	RH	shine hrs	mm	mm
2005	20.08	10.90	59.23	54.15	345.80	2860.80	3.08
2006	20.18	11.83	55.12	46.25	343.80	2730.20	2.94
2007	19.86	11.19	57.61	45.54	359.30	1848.60	3.09
2008	19.65	10.88	56.90	47.20	328.40	2304.80	2.86
2009	20.47	10.61	59.49	45.84	348.00	1768.40	2.97
2010	25.62	13.52	73.57	56.23	2410.6	2569.6	3.48
2011	23.90	13.17	81.31	67.24	331.41	2500.60	2.89
2012	24.01	12.81	70.96	56.18	348.23	2421.99	3.42
2013	23.55	13.05	75.55	61.18	328.41	3142.00	2.80

Some parameters were also recorded like GPS readings, plant height, Diameter and soil samples, leaf samples and also stem cuttings for vegetative propagation. However, some plants do not have stem cuttings (Fig.1) and in semi dried condition and some of them are very young. The semi hard stem cuttings were selected for vegetative propagation, the average length of

cuttings was 15-20 cm long and 6.0 to 9.0 mm diameter with 5 to 6 nodes. The stem cuttings were collected in the last week of November to middle December. Some growth hormones and phenolic acids were selected for rooting. The material, (stem cuttings) was also collected from Mussoori, Dehradun, Ranikhet, Nanital (Uttrakhand) Shimla, Manali, Kalpa (H.P.) and Srinagar (J&K) for comparison regarding quality parameters.

Fig 1

Fig 2

Fig 3

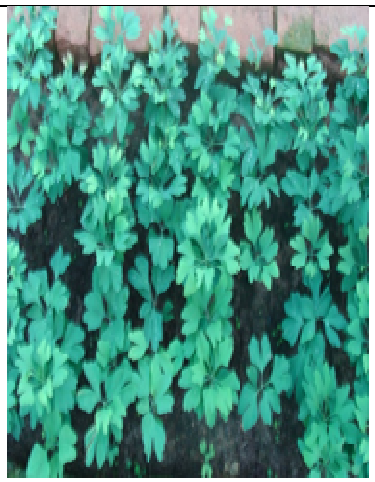

Fig 4

Fig 5

Fig 6

Fig 7

Fig 8

Fig. 1. Shows, a near extinct condition of the *G. biloba* tree. **Fig. 2.** Sprouted semi hard cuttings of *G. biloba* after one year. **Fig. 3.** Sprouting of semi hard cuttings after 2 years. **Fig. 4 and Fig. 5.** Seedlings transferred in the poly sleeves by seeds raised. **Fig. 6.** Rooted seedlings raised by seeds. **Fig. 7.** A field trial (FYM x Spacing) in dormant period. **Fig. 8.** A field trial in fully growth season (July-August).

The rooting experiments were planned separately using different concentrations of hormones 250, 500 and 1000 mg/l of each alone and combined to study the cumulative effects of combinations. The combinations IBA + Phloroglucinol (250mg/l+10mg/l), (250mg/l+20mg/l), (500mg/l+10mg/l), (500mg/l+20mg/l), (1000mg/l+10mg/l), (1000mg/l+20mg/l), NAA 250mg/l, 500mg/l and 1000mg/l, IAA 250mg/l, 500mg/l and 1000mg/l, Ascorbic acid 25mg/l, Tanic acid 2.5mg/l Tanic acid 5mg/l and 10mg/l Catchin acid 2.5mg/l, 5mg/l and 10mg/l, Galic acid 2.5mg/l, 5mg/l and 10mg/l were used. All hormones were dissolved in distill water (v/v) aqueous ethanol; untreated cuttings were dipped in distilled water. All the semi hard stem cuttings were dipped in different hormones/ phenol for two hours at 20^0C. In the present experiment 16 treatment combinations (16x3) were made. Out of which each treatment consists of 150 number cuttings (Table 2) with three replications (50 cuttings per replication). Mean for each treatment was based on observations.

Table- 2. The effect of different hormones and phenolics of the sprouting of semi hard stem cuttings of *G. biloba*.

	Treatment	Stem cutting sprouting (%)	Rooting (%) 1st year	Rooting (%) 2nd year	Average roots/plant (No.)	Average Root length/plant (cm)
1	IBA + Phloroglucinol 250mg/l+10mg/l	70.00	60.00	66.00	7.00	12.33
2	IBA + Phloroglucinol 250mg/l+20mg/l	63.33	56.67	59.33	6.67	11.33
3	IBA + Phloroglucinol 500mg/l+10mg/l	66.67	63.33	64.67	6.33	11.33
4	IBA + Phloroglucinol 500mg/l+20mg/l	53.33	43.33	47.33	5.33	10.67
5	IBA + Phloroglucinol 1000mg/l+10mg/l	56.67	46.67	52.67	5.33	12.00
6	IBA + Phloroglucinol 1000mg/l+20mg/l	40.00	23.33	32.67	4.00	11.33
7	IAA- 250mg/l	75.33	71.33	74.00	7.00	15.33
8	IAA- 500mg/l	70.00	61.33	66.00	6.00	13.33
9	IAA- 1000mg/l	62.67	54.00	59.33	5.00	11.00
10	IBA- 250mg/l	91.33	79.33	85.33	7.00	13.67
11	IBA- 500mg/l	72.67	69.33	68.67	5.33	12.67
12	IBA- 1000mg/l	72.00	61.33	66.67	4.67	12.33
13	NAA- 250mg/l	83.33	74.00	80.67	6.33	14.67
14	NAA- 500mg/l	75.33	60.67	62.00	5.33	12.33
15	NAA- 1000mg/l	50.00	44.00	46.67	4.00	11.67
16	Ascorbic acid 25mg/l	40.00	30.67	34.00	6.33	10.67
17	Ascorbic acid 50mg/l	57.33	52.00	54.67	7.33	11.00
18	Ascorbic acid 100mg/l	61.33	55.33	56.67	7.67	11.67
19	Salicyclic acid 15mg/l	28.67	22.67	26.67	6.67	12.00
20	Salicyclic acid 20mg/l	44.67	36.00	40.00	7.00	12.33
21	Salicyclic acid 25mg/l	57.33	47.33	52.67	7.33	13.00
22	Tanic acid 2.5mg/l	24.00	23.33	23.33	4.67	12.67
23	Tanic acid 5mg/l	35.33	26.00	32.67	5.67	13.33
24	Tanic acid 10mg/l	33.33	30.00	31.33	6.00	14.00
25	Catcin acid 2.5mg/l	90.00	68.67	74.67	5.33	8.73
26	Catcin acid 5mg/l	93.33	80.00	84.67	5.33	10.67
27	Catcin acid 10mg/l	96.67	58.67	58.00	5.67	11.00
28	Galic acid 2.5mg/l	86.67	66.67	71.33	4.67	9.33
29	Galic acid 5mg/l	90.00	54.00	62.67	4.33	8.67
30	Galic acid 10mg/l	93.33	62.67	69.33	5.67	8.00
31	Control (without treatment	30.00	18.00	23.33	3.00	10.00
	CD (P=0.05)	21.00	21.41	20.04	2.35	3.15

The seeds of *Ginkgo biloba* were procured from m/s Yu Wangsheng, Wuhan All Green seed, 27-2-3-301, Changqing Garden, Wuhan 430023, China; in the month of October 2007. Seeds were stored in an incubator at 5^0C for one month. The seeds were dipped in ordinary water for five to six minutes; the seeds were washed and cleaned with a clean towel and all the water droplets were removed from the outer layer of the seed coat. The seeds were again kept in an incubator at 5 ^{0}C. This process was applied after every 10 days of interval. Before sowing, the old soil from beds has been removed up to 8". A 2" layer of fine sand was spread in deep surface of the beds. In the middle layer 1:1:1 ratio (One part garden soil: One part FYM: One part sand) were thoroughly mixed and spread a 4" layer above at the fine sand layer. Again at the top a 2" fine sand layer was also spread out. The seeds were sown at 2" depth in the sand layer. The seed germination trials were laid out on 27-12-2007 (Fig.3 and Fig.4). The hormones concentration of IAA, IBA, NAA and Salicyclic acid 250mg/l, 500mg/l and 1000mg/l were taken and the seeds were dipped for one hour time duration. Ninety number seeds were used for each concentration (Table 3). In the control the same number i.e. 90 number seeds were taken and dipped in water for one hour. One set were sowing in poly-house and 2nd in open bed just covered a 50% agro-net for partial shed only at 3 feet

height. The irrigation was done as or when it required. Outside in the open bed, we have used only 1:1:1 ratio of soil texture (One part garden soil: One part FYM: One part fine sand) the same process was applied as in the previous case. The seeds were sown only at 2" depth. The first germination of *G. biloba* seeds appeared to date 17.03.2008 and continued up to 30th April 2008 about 32.27 to 85.74 % seed germination was recorded.

Table 3: Seed germination (%) and growth performance of seeds raised saplings after germination under the treatment of different hormones.

	Treatment	Seed Germination %	Leaves No. DAG* (0 to7)	Leaves No. DAG* (8 to15)	Leaves No.DAG* (16 to 22)	Plant height (cm) DAG* (0 to7)	Plant height (cm) DAG* (8 to15)	Plant height (cm) DAG* (16 to 22)
1	IAA - 250 mg/l	70.49	1.67	2.67	5.33	1.17	5.50	13.83
2	IAA - 500 mg/l	82.35	2.00	3.00	5.67	1.67	6.83	13.67
3	IAA - 1000 mg/l	82.69	2.00	2.67	5.67	1.83	4.83	13.83
4	IBA - 250 mg/l	85.74	1.33	3.00	5.33	1.50	6.17	14.67
5	IBA - 500 mg/l	79.97	2.00	3.00	5.67	1.50	6.50	14.83
6	IBA - 1000 mg/l	72.18	1.33	2.67	5.33	1.67	7.00	16.67
7	NAA - 250 mg/l	78.95	1.67	3.00	5.00	1.17	6.67	16.67
8	NAA - 500 mg/l	84.73	1.67	2.67	5.67	1.00	7.50	15.17
9	NAA - 1000 mg/l	73.20	2.33	3.33	5.67	1.83	6.67	13.17
10	Sal.Acid - 250 mg/l	81.68	2.00	2.67	6.00	2.00	6.33	12.83
11	Sal.Acid - 500 mg/l	73.55	1.67	2.33	5.67	1.83	5.67	12.17
12	Sal.Acid - 1000mg/l	69.16	1.33	3.67	5.67	1.83	6.33	13.50
13	Control (without treatment)	32.27	1.00	2.00	4.33	0.47	3.83	10.67
	CD(P=0.05)	1.83	NS	NS	NS	0.76	NS	NS

Table 4: Vertical growths of seeds raised saplings under the treatments of different hormones.

S. no.	Treatments	Plant height (cm) June-2008 at the time of transplanting in sleeves	Leaves No. June-2008 at the time of transplanting in sleeves	Roots length (cm) June-2008 at the time of transplanting in sleeves	Roots no./plant June-2008 at the time of transplanting in sleeves	Plant height in(Cm) May-2009	Plant height in (Cm) May-2010	Plant height in (Cm) January-2011
1	IAA - 250 mg/l	18.75	11.58	17.83	9.50	34.67	70.33	80.50
2	IAA - 500 mg/l	19.00	13.08	18.42	10.17	40.50	66.67	81.17
3	IAA - 1000 mg/l	17.58	13.50	18.17	10.83	40.67	68.67	90.00
4	IBA - 250 mg/l	18.83	13.83	19.17	10.50	42.17	102.67	117.00
5	IBA - 500 mg/l	19.50	14.25	16.58	10.83	39.33	103.33	115.00
6	IBA - 1000 mg/l	19.75	13.25	18.50	12.83	35.50	104.67	118.67
7	NAA - 250 mg/l	17.08	12.17	12.83	9.00	38.83	104.33	118.00
8	NAA - 500 mg/l	18.00	12.58	13.50	9.50	41.67	97.33	116.67
9	NAA - 1000 mg/l	16.67	10.42	14.58	11.50	36.00	97.00	115.67
10	Sal.Acid - 250 mg/l	18.33	11.08	13.58	8.50	40.17	92.00	110.33
11	Sal.Acid - 500 mg/l	18.08	12.00	14.92	8.83	36.50	91.67	111.33
12	Sal.Acid - 1000mg/l	18.50	11.25	15.00	9.33	34.00	91.33	104.33
13	Control (without treatment)	10.73	6.33	7.93	5.50	15.87	36.63	52.07
	CD(P=0.05)	2.24	2.10	0.95	1.38	9.31	5.94	7.83

The germination started after 128 days and continued up to 192 days. The agro-climatic conditions of Palampur during, the month of November to February, is extremely cold. After germination, the percentage of germination and leaves number were recorded. At the time of transplanting, plant height, number of roots and root length were also recorded (Fig. 6). The growth performance and relative growth rate of different treated hormones was also recorded from June 2008 to January 2011.

Table 5: Vertical growth of *Ginkgo biloba* under the treatment of different doses of F.Y.M. in different year's (in six year).

	Treatment	Plant height (cm) 2005	Plant height (cm) 2006	Plant height (cm) 2007	Plant height (cm) 2008	Plant height (cm) 2009	Plant height (cm) 2010	Plant height (cm) 2011	Plant height (cm) 2012	Plant height (cm) 2013
1	FYM 15t/ha	67.60	84.67	111.85	134.77	194.60	233.67	269.40	295.63	289.93
2	FYM 30t/ha	57.92	74.80	96.52	116.43	175.76	220.06	261.44	290.21	305.93
3	FYM 45t/ha	63.50	82.30	105.83	129.68	185.13	224.84	260.73	299.15	320.43
4	FYM 60t/ha	55.79	80.70	103.75	129.98	194.79	238.27	277.85	310.79	325.08
5	FYM 0t/ha	44.20	63.20	77.60	89.00	135.10	186.60	235.00	257.60	275.80
	CD(P=0.05)	8.05	6.81	11.60	10.27	9.82	21.63	16.24	33.01	30.24

Table 6: Radial growth of *Ginkgo biloba* under the treatment of different doses of F.Y.M. in different year's (in six year).

Plant circumference (cm) at ground level year wise										
	Treatment	2005	2006	2007	2008	2009	2010	2011	2012	2013
1	FYM 15t/ha	2.67	3.92	6.62	8.97	13.97	18.83	22.47	75.46	76.37
2	FYM 30t/ha	2.48	3.81	6.33	8.49	12.56	17.81	20.65	72.67	79
3	FYM 45t/ha	2.92	4.49	6.70	9.14	13.62	18.50	21.19	77.22	82.53
4	FYM 60t/ha	2.76	4.18	6.84	9.38	14.14	18.83	21.12	78.00	86.02
5	FYM 0t/ha	2.72	3.66	4.94	6.04	9.34	14.80	17.40	62.40	67.60
	CD(P=0.05)	NS	0.30	0.69	1.44	1.52	1.83	2.32	10.76	8.05

Table 7: Plant circumference at the Brest height (BDH) of *Ginkgo biloba* at different treatment of F.Y.M. and their fresh leaves weight.

	Treatment	Plant circurfrance (cm) at brest height 2009	Plant circurfrance (cm) at brest height 2010	Plant circurfrance (cm) at brest height 2011	Plant circurfrance (cm) at brest height 2012	Plant circurfrance (cm) at brest height 2013
1	FYM 15t/ha	6.10	9.57	12.81	44.43	47.43
2	FYM 30t/ha	5.20	8.30	11.53	41.19	49.23
3	FYM 45t/ha	4.47	7.63	10.88	44.89	51.57
4	FYM 60t/ha	5.77	8.80	12.22	46.01	53.20
5	FYM 0t/ha	2.40	5.40	9.20	35.20	44.80
	CD(P=0.05)	2.07	2.25	2.24	NS	NS

Table 8: Fresh weight of leaves of *Ginkgo biloba* t/ha in consequent five years in different FYM application.

Treatments		Leaves fresh weight t/ha 2009	Leaves fresh weight t/ha 2010	Leaves fresh weight t/ha 2011	Leaves fresh weight t/ha 2012	Leaves fresh weight t/ha 2013
1	FYM 15t/ha	0.834	1.098	3.338	2.34	1.04
2	FYM 30t/ha	0.910	1.403	2.489	1.88	1.11
3	FYM 45t/ha	0.865	1.255	3.494	2.55	1.19
4	FYM 60t/ha	0.744	1.398	3.090	2.28	1.27
5	FYM 0t/ha	0.429	0.723	0.712	0.50	0.47
	CD(P=0.05)	0.260	0.432	1.259	0.88	0.08

Table 9: Dry weight of leaves of *Ginkgo biloba* t/ha in consequent five years in different FYM application.

Treatments		Leaves dry weight t/ha 2009	Leaves dry weight t/ha 2010	Leaves dry weight t/ha 2011	Leaves dry weight t/ha 2012	Leaves dry weight t/ha 2013
1	FYM 15t/ha	0.384	0.505	1.536	1.08	0.53
2	FYM 30t/ha	0.419	0.646	1.145	0.86	0.57
3	FYM 45t/ha	0.398	0.577	1.607	1.16	0.61
4	FYM 60t/ha	0.342	0.643	1.421	1.02	0.65
5	FYM 0t/ha	0.197	0.332	0.328	0.26	0.24
	CD(P=0.05)	0.120	0.199	0.579	0.51	0.04

A field trial was also laid out by using four different doses of FYM, 15 t/ha, 30 t/ha, 45 t/ha and 60 t/ha were taken and as per calculation per pit FYM was given. The pit size was 60 x 60 x 60 cm, the FYM doses were given before planting and every year in the month of December. The experimental plantation was done during January 2005, a total no. of 315 plants were planted; in five treatments of 63 plants each.

In all the treatments plants were planted at the distance of 3m x 3m plant to plant and row to row. At the time of planting, soil samples of top soil and sub-soil were taken and analyzed for physico-chemical properties. The soil was silty clay loam in texture, normal to slightly acidic in soil reaction with high organic carbon percentage. Available N,P&K high and low.

The observation of plant height and diameter were recorded monthly, at ground level. The observations were started from April 2005 and recorded annually up to 2013. The FYM doses were also given in December every year. The breast height diameter was also recorded in last five years i.e. 2009 to 2013. The leaves were also harvested in last five years for purpose of commercial use. All the parameters were statistically analyzed under the statically program of STATISTICA-7.

4. Result

4.1 Study on semi hard stem cuttings

In terms of sprouting percentage of semi-hard stem cuttings of *G. biloba,* rooting behaviours of cuttings treated with different concentrations of IBA along with phloroglucinol (Fig.2,Fig.3), IBA (500 mg/l + phloroglucinol 10 mg/l) recorded statistically significant highest percentage (63.3%) of rooting followed by IBA (250 mg/l + phloroglucinol (10 mg/l) in the first year. In terms of rooting (%) and average roots/plant in first and second year, the results as recorded were statistically significant (Table 2). However, the growth hormones treated singly with different concentrations IAA, IBA and NAA 250, 500 and 1000mg/l produced statistically significant sprouting and rooting percentage in the first and second year (Table 2). In comparison to IAA, NAA and IBA 250 mg/l produced highest sprouting percentage (91.33%). Surprisingly catchin acid and Galic acid produced statistically significant sprouting percentage, rooting percentage in first and second year simultaneously in comparison to control (Table 2). Besides, the main treatment we have tried to compare the sprouting percentage in soft stem cuttings and hard cuttings in our separate experiment. In soft cuttings the sprouting percentage is very less, in comparison to hard cuttings. However, best results were observed in the cuttings taken from three year old side branches which were more than pencil thickness.

4.2 Study on seed germination

The experiment of seed germination was laid out to study the effect of hormones on seed germination. In Palampur climatic conditions, the dormant period is start from November onwards to middle March (Fig.7). The parameter of seed germination was recorded from the seed sowing and starting of seed germination after 128 days and up to 192 days (Table 3). The maximum statistically significant seed germination was recorded in the treatment of IBA in 250 mg/l (85.74%). In NAA 500 mg/l the germination percentage was (84.73%), but it was comparable to the IBA 250 mg/l and the parameters was statistically significant. The higher concentration of IAA 500 mg/l and 1000 mg/l, the germination percentage was statistically significant in comparison to 250 mg/l, but higher concentration are comparable. The treatments of IBA, NAA and Salicylic acid statistically significant each other in terms of concentration. In comparison to control all the hormonal treatment were statistically significant. The leaves and plant height were also recorded up to 192 days, at weekly interval. Only plant height was statistically significant (Table 3). The growth parameters after the transfer of one year old plants in poly sleeves for hardening up to January 2011 (Table 3). The plant height, leaves number, root length and roots number/plant were recorded at the time of transfer and recorded up to January 2011 (Table 4). The new sprouting starts in the last week of March. It is observed that the growth of *G. biloba* occurs during April-May only in a rhythmic growth pattern yearly, (Fig.8). It happens when the dormant period is over. At the same time the root length and root number/plant recorded, when the saplings were transferred in the sleeves i.e. in the last week of June 2008 (Table 4). The roots number/plant was also statistically significant in between the hormonal treatments as well as in comparison to control. In control the root length and roots number/plant was very low in number and less as compared to all other treatments. (Fig.6).

In last eight to ten years, as we standardized the vegetative propagation technique in semi hard stem cuttings of *G. biloba*. We have raised more than 30,000 (thirty thousand) number of saplings. It is a rare and endangered medicinal plant. We have planted in five acre area (under different R&D experiment) in CSIR-IHBT, Palampur. Biodiversity garden (Fig.7 and Fig.8) for chemical evaluation under different R&D experiment. Besides, we have distributed about 8000 (eight thousand) number of plants to the farmers, forest department, state governments, NGO's and industrialists. We are keeping track of the plantation done in different areas. Still, we have 30,000 (thirty thousand) plants five to six years old from semi hard stem cuttings.

4.3 Study (FYM x spacing) in field experiment

In case of field trial, the FYM doses were used @ 15, 30, 45 and 60 t/ha. The plant height, relative growth rate/year was statistically significant in comparison to control. The lower doses of FYM 15t/ha has produced better plant height in comparison to higher doses (Table 5). In terms of plant circumference also the results are statistically significant in

lower doses of FYM 15 t/ha (Table 7). In last five years 2009 to 2013 the plant height at breast measure and circumference were also measured (Table 7). The findings indicate statistically significant results of FYM application in comparison to control. In the field experiment (FYM x spacing), the fresh leaves was harvested in five consequent years viz. 2009 to 2013 in the month of November every years (Table 8). However, during the year 2009, 10 to 15% leaves were damaged due to hail storm during month of May. In the last two years viz 2012 and 2013 again heavy hail storm during the month of April, May and September heavy damage about 40 to 50 percent of leaves and tear-of many fold. In the year 2009 and 2010, FYM dose 30t/ha showed batter performance (fresh weight of leaves) while in 2011, 45t/ha was excellent and produced statistically significant fresh weight (Table 8). In the year 2012 and 2013 the fresh and dry weight of leaves simultaneously decreased.

5. Discussion

The overall purpose and objective was to standardize the propagation and agro techniques by using semi hard stem cuttings and through seed germination and also established cultivation field practices for batter leaf production of this commercially important plant species. Studies were made regarding the effect of some growth hormones and phenolic acid, including Indole acetic acid (IAA), Indole butyric acid (IBA), Naphthalene acetic acid (NAA) and Salicylic acid on seed germination and sprouting of semi hard stem cuttings percentage, relative growth rate (RGR), plant height, leaves number, root length and their number and rooting percentage. And a field experiment using different (FYM) farm yard manure doses 15t/ha, 30t/ha, 45t/ha and 60 t/ha application, studies in terms of RGR, plant growth, plant height and diameter at ground level and at breast height, ultimately the fresh leaf biomass production in consequent during five years. It was recorded that all the applied concentrations of growth hormones and salicylic acid had statistically significant and promoting effects on seed germination as well as on sprouting/bud break of cuttings in comparison to control. It was also observed that as the doses of FYM increased the plant height and diameter significantly increased as compared to control.

It has been reported that when the seeds were sown in warm places, they germinate within 33 days and in cold place it takes more time (http://www.worldagroforestrycentre.org), however, in our case the seeds were sown in cold climatic conditions i.e. in the month of December which is the extreme cold period here. The germination period was 128 days. Our results fully agreed with the above i.e. *G. biloba* has a strong correlation with meteorological factors. They have also reported that IBA and IAA are the best growth regulators in terms of seed and vegetative propagation. These finding agreed with our results. A stratification period of thirty to sixty days at 5 ^{0}C before sowing seeds has been recommended (Ponder *et al*., 1981; Dirr and Heuser, 1987; Willan, 1985) [30, 8, 44]. The process as adopted by us before seed sowing fully agreed with above quoted findings.

In the response of pretreatment of semi hard stem cuttings with growth hormones and phenolic acids, especially auxins, the endogenous level of auxins has increased and its availability in the surface area of cut ends which in turn, help in rooting (Krishnamoorth, 1981) [21]. Auxins alone or with combination of phenolic compounds have also been observed that they stimulate adventitious root formation in stem cuttings of many species (Kling and Meyer, 1983**;** Nandi *et al.*, 1997) [19, 26]. The same case may be assured in the case of seeds, it was observed that when the pretreatment of growth hormones and the endogenous level of hormones are increased they ultimately stimulate the seed germination and rooting. However, the promotory effects of auxins during root initiation are very well reported (Haissag, 1986) [11].

These compounds function via activation of enzyme mobilization of food materials leading to cell division, cell elongation and successful embryo growth and hence germination of in viable seeds (Maguire, 1975; Khan, 1980) [22, 17]. EL – Sharkawi and Springuel, (1979) [9] found that IAA influences the elongation phase more than emergence phase of both plumule and the radical of germinating seeds. IAA and IBA increased the plant height, leaf number, cell division, cell elongation and chlorophyll synthesis (Mukaila *et al.*, 1996- 97) [24].

These compounds function may be cumulatively via activation of enzymes, mobilization of food materials leading to successful embryo growth and hence germination in viable seed especially when dormancy barrier has been overcome.

Stimulation of plant height, leaves number, root length and their number by growth regulators at low concentrations has also shown in so many tree species and vegetables (MuKaila et.al. 1996-97) [24].

The relationship between endogenous hormone with growth and drops in ginkgo seeds were studied (Wang, *et al.*, 2001) [42]. Kramer and Bennett, (2006) [20] have reported that auxin moves between plant cells through combination of membrane diffusion and carrier-mediated transport.

In the seed treatment of growth regulators and salicylic acid, it is clear that the growth regulators play an important role in the stimulation of seed germination. IAA, IBA and NAA treatment are clear cut indications showing that all the used concentration has promoted statistically significant seed germination percentage at a great extent as compared to the control.

In the case of semi hard stem cuttings, it has been observed that some young plants of *G. biloba* bear good stem cuttings resulting in better sprouting percentage. While stem cuttings collected from old trees gave poor sprouting and dies very soon. (fig.1). On the basis of our results sprouting percentage of semi hard stem cuttings from three year old side branches, it may be assumed that a certain amount of food reserves or secondary metabolites were sufficient at this stage for appropriate sprouting of stem cuttings.

Response to application of nitrogen and phosphorus has also been studied on the growth of *G. biloba*. N fertilizer allowed *G. biloba* trees to maintain typical foliar N-dynamics even during a persistent drought during which foliar P dynamics were significantly altered (Son- Yowhan and Son- Y, 2002) [39]. The effect of fertilizer on growth and leaf yield in ginkgo leaf producing plantation were also studied (Xie- Youchao, *et al.*, 2000) [45]. Del-Tredici, (1992) [7] has studied the natural regeneration of the *Ginkgo* plant from cotyledonary.

G. biloba is an eco-friendly tree (Neinhuis & Barthlott, 1998) [27] and having potential to grow even in habitat polluted by industrial pollutants such as SO_2 (Huang *et al.* 1990) [12]. Xing *et al.*, (1997) [46] has examined and reported the analysis of the nutritional components of seed of promising *Ginkgo* cultivars.

From these points in the support of *G. biloba,* it becomes imperative that we should protect and preserve it in nature. According to our survey report a very few numbers of *Ginkgo* plants are surviving in our country (Gopichand *et al.*, 2009) [23]. To study the quality of available *Ginkgo* plants in India, some Ginkgolide A, B,C,J and bilobalide (BB) studied (Kaur *et al.*, 2009) [15]. For the conservation purpose, we have selected twelve clones from all over India on quality basis

(Kaur *et al.*, 2012) [16]. We have propagated vegetatively by semi hard stem cuttings and established in our Biodiversity farm in the institute. In the same way, the estimation of genetic diversity in *G.biloba* has also been studied (Kumar *et al.*, 2010). On the bases of surveys the leaves samples were collected and studied the altitudinal chemical changes in leaves (Kaur *et al.*, 2012) [16].

For rapid and better growth performance of *G. biloba* propagation by semi hard stem cuttings and use of certain growth hormones are effective (Cheng *et al.*, 1996; Van-Staden *et al.*, 1983; West *et al.*, 1970) [5, 41, 43]. Owing to the high demand of ginkgolide-B and other compounds, micro-propagation has also been attempted by using leaves as explants (Jenn *et al.*, 1995; Chen-Xue- Sen *et al.*, 1997; Carrier *et al.*, 1990) [14, 3, 2].

Chen *et al.*, (1997) [3] have reported on the cultivar selection in *G. biloba*. The history of *G. biloba*, leaf resources, quality maintenance for production of extracts, active constituents, ethno-pharmacology properties and prospect for the conservation and use of this medicinal plant have been studied in detail in China (Pang *et al.*, 1996) [29]. Zhang and Zhang, (1994) [47] have reported that it can be cultivated at altitudes up to 2000 m. and leaves can be used for the production of commercial products by pharmaceutical industries. The concentrations of ginkgolides and bilobalides in leaves in relation to the seasonal variations have been determined (Beek *et al.*, 1992; Kaur *et al.*, 2012) [1, 16].

Roberts, (1972) [35] has concluded that depletion of essential metabolites, including loss of food reserves, is one of the important factors responsible for loss of seed viability. Prolonged moist storage leading to fungal infection might be responsible for further loss of viability (King and Roberts, 1979) [18].

Currently it is a million dollar business all over world especially in European countries to multiply this species of impressive long living, commercial and most preferred avenue plantation.

6. Conclusion

In the present studies, the main objective was to standardize the propagation techniques by using semi hard stem cuttings and through seed germination under the effect of some growth hormones and phenolic compounds. In the case of semi hard stem cuttings and seed germination, the IBA was produced the most significant results. Surprisingly catchin and galic acid also produced significant sprouting and rooting percentage. An experiment was also laid out in the field (FYM x spacing) to study the growth parameters and ultimately the leaf production in each treatment for commercial importance. In the field experiment (FYM x spacing), in terms of growth parameters lower FYM dose (15 t/ha) produced significant results. Whether, in terms of leaf production FYM dose (30 t/ha) gave significant results.

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