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Callus-mediated organogenesis in *Lilium polyphyllum* D. Don ex Royle: a critically endangered Astavarga plant

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***Lilium polyphyllum* D. Don ex Royle (Liliaceae) is a critically endangered herbaceous perennial, commonly known as white lily or Ksheerkakoli. Bulbs of the plant are of immense medicinal use and have astringent and anti-inflammatory properties. Overexploitation of the species from the wild and degradation of habitats are posing threats to its existence. In the present study, a protocol was standardized for micropropagation and mass multiplication of the species from scale leaves. Callusing was induced in basal MS medium containing 2,4-D (6.78 µM) and BAP (4.4 µM), where maximum effect (95.32%) was recorded. Maximum shooting (97.45%) was found in the calluses when shifted to MS medium fortified with BAP (4.4 µM), NAA (0.53 µM) and GA_3 (20 ppm) with an average of 19.2 shoots/per culture. The well-developed *in vitro* regenerated shoots were shifted to the rooting medium and 100% rooting was achieved in half-strength MS basal medium enriched with IBA (9.8 µM). The *in vitro* regenerated plantlets were shifted to a glasshouse for acclimatization and finally transferred to the open environment with 85% success.**

Keywords: Callusing, *Lilium polyphyllum*, micropropagation, organogenesis.

LILIUM POLYPHYLLUM, a bulbous perennial herb of the Liliaceae family, is commonly known as white lily and Ksheerkakoli. IUCN has categorized it as critically endangered medicinal herb and included it under the Red Listed species[1]. It commonly grows in dense, humus-rich forest floors at an altitudinal range 2100–3000 m. The plant grows up to 1.0–1.5 m height, produces white–creamy flowers with purple spots and triangular capsules bearing winged seeds[2,3]. Distribution of the species is restricted to the Himalayan region and it is sparsely distributed from Afghanistan to North-West Himalaya in India. In the Indian Himalaya, the species is surviving with a few populations in Himachal Pradesh (Pulga–Kullu, Dhauladhar and Shimla), Jammu and Kashmir (Chatru and Doda) and Uttarakhand (Chakisain, Gargia-Pithoragarh, Chakrata, Raithal Harsil, Gangotri, Valley of Flowers, Kaddukhal and Dhanaulti)[1–6]. Bulbs are used for

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the preparation of various traditional and modern medicines and have an increasing demand in traditional and pharmaceutical industry. They possess soothing, astringent, refrigerant, galactogogue, expectorant, aphrodisiac, diuretic and anti-inflammatory properties, and are being used for the cure of fever, cough, bronchitis, seminal weakness, burning sensation, hyperdipsia, hematemesis, strangury, etc.[7]. Paste of bulb is one of the main components of revitalizing creams and Chywanaprasha (an ancient herbal preparation)[8]. This species is one of the eight herbs of the Astavarga medicinal system and in the local and national markets, the bulbs are traded under the name Kakoli/Ksheerkakoli. Bulbs contain linalool and α-terpineol as the main chemical constituents[9]. Natural regeneration of the species takes place either by vegetative propagation with the help of underground perennating bulbs or by seeds.

The unabated trade, ruthless degradation of habitat by grazing[10] coupled with global warming/climate change and infestation of habitat by invasive species has caused shrinking of the species in the wild. The situation is further aggravated by the epicotyls-morphophysiological seed dormancy in *L. polyphyllum*[11]. Due to dwindling population of the species in the wild, it has resulted into the threatened status in the Red Data Book of Indian Plants[12] and subsequently as critically endangered by IUCN[1]. Rana and Samant[13] advocated *in situ* and *ex situ* conservation measures for *L. polyphyllum*. Dhyani *et al.*[14] attempted propagation of the species through seed germination and *in vitro* bulbet formation from callus with the help of plant growth regulators (PGRs).

In view of the available literature and critically endangered status of the species, the present study was aimed to standardize an efficient and reproducible micropropagation protocol for mass multiplication of the species through callus-mediated organogenesis.

Mature and healthy bulbs were collected in October from Kaddukhal (2300 m amsl, 30°24 N, 78°17 E), Tehri district, Uttarakhand. Initially the bulbs were washed in running tap water for 30 min and followed by Savlon (Johnson and Johnson), a germicide and 1.0% solution of Tween-20 (Himedia Laboratories, India) in 150 ml water for 20 min. Each treatment was followed by thorough rinsing in running tap water to remove the detergent completely. Individual scale leaves were segregated from the bulbs and surface-sterilized with 70% ethanol for 10 min followed by treatment of 0.1% $HgCl_2$ for 10 min. After each treatment, the scales were thoroughly rinsed with autoclaved double-distilled water. Scales were inoculated in basal MS medium[15]. Cultures were maintained in the culture room at 24 ± 2°C, under a 16/8 h light and dark cycle with light intensity of 47.29 μmol m^{-2} s^{-1} provided by white fluorescent LED tubes (40 W; Wipro, India).

The scales were excised into small pieces (0.5 cm) and inoculated in MS medium fortified with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (2.26–9.0 μM) and subsequently, the optimal concentration of 2,4-D (6.7 μM) was tested along with different concentrations of cytokinins, viz. 6-benzylamino purine (BAP) (1.3–6.6 μM), kinetin (1.39–6.9 μM) and thidiazuron (TDZ) (1.36–6.81 μM). Callus cultures were maintained by subculturing at an interval of four weeks. The callus was further proliferated into the callus proliferation medium.

The *in vitro* raised calluses were shifted to the shooting medium. The shoot induction medium was enriched with different concentrations of cytokinins, viz. BAP (2.22–8.9 μM), TDZ (1.36–6.81 μM) and kinetin (2.32–9.3 μM). Subsequently, the optimal concentration of BAP (4.4 μM), TDZ (4.54 μM) and kinetin (6.9 μM) was further tested along with different concentrations of naphthaleneacetic acid (NAA) (0.53–1.59 μM). One set of calluses were also transferred into half-strength MS medium containing optimized concentration of BAP, NAA and gibberellic acid (GA_3) (20 ppm).

Shoot cultures were maintained by subculturing at regular intervals of four weeks. Data were recorded for the percentage of explants with a positive response, number of total shoots per explant and shoot height to calculate the shoot proliferation rate after six weeks of incubation.

Clusters of 2–4 shoots (8–10 cm length) were transferred to various root induction media. Initially, the shoots were cultured on MS and modified MS media (half, quarter and zero salt strength). Subsequently, the basal media of different salt strengths were supplemented with various auxin concentrations (Himedia Laboratories, India), viz. indolebutyric acid (IBA) (2.46–12.26 μM), indoleacetic acid (IAA) (2.85–14.27 μM) and NAA (2.65–13.25 μM) individually. The half-strength MS medium was found most suitable for growth of *in vitro* roots, followed by quarter and full-strength MS media fortified with IBA (9.8 μM). Cultures were maintained in similar media and data were recorded for rooting percentage, number of roots and root length after two-weeks of incubation.

After incubation for four weeks in rooting medium, the *in vitro* raised healthy plantlets were removed from the culture tubes and washed gently under running tap water to detach the medium from the roots. *In vitro* raised plantlets were shifted to plastic pots containing vermiculite and sterile soil in 1 : 1 ratio. To ensure optimum humidity, pots were covered by transparent polythene sheet and were provided half-strength modified Hoagland solution[16] at an interval of three days. Polythene sheet was removed after two weeks in order to acclimatize the plants to the open environment. After successful acclimatization to open environment, the plantlets were transferred to pots in the greenhouse.

All the experiments were conducted in triplicate. The effects of the different treatments were recorded, and the

level of significance was determined by analysis of variance using SPS software.

The scales inoculated in MS medium (control) did not show any callusing. Incorporation of different concentrations of 2,4-D (2.26–9.0 μM) initiated callus induction in scale explants and 65.40% callusing was achieved in 2,4-D (6.7 μM). Further, supplementing the medium with varying concentrations of cytokinins, viz. BAP (1.3–6.6 μM), TDZ (1.36–6.8 μM) and kinetin (1.39–6.9 μM) was found to enhance the callus induction percentage and 95.32%, 90.07% and 87.11% callusing was reported, respectively. Among the three cytokinins used, BAP was found most effective and 95.32% callusing was reported in MS medium containing 2,4-D (6.78 μM) and BAP (4.4 μM). Callus obtained was yellow, globular and friable in texture (Table 1 and Figure 1 *a*). It was further proliferated in the same medium.

In the MS medium (control), frequency of shoot induction in callus was negligible and no morphogenetic response was reported. Incorporation of BAP (2.2–8.9 μM), TDZ (1.36–6.8 μM) and kinetin (2.32–9.3 μM) separately into the medium enhanced the shoot multiplication rate up to 65%, 60% and 58% respectively (Table 2). Different concentrations of BAP, TDZ and kinetin were tested, 4.4 μM BAP, 4.54 μM TDZ and 6.9 μM kinetin proved to be the most effective. Since these yielded the optimum shooting percentage, they were further tested along with varying concentrations of NAA (0.53–1.59 μM), in which significant enhancement was found in the multiplication rate and average shoot length. BAP (4.4 μM) and NAA (0.53 μM) proved to be the most optimum concentration for *in vitro* shoot induction in callus explants and 97.45% shooting was achieved with 19.2 average shoots per culture and 8.6 cm shoot length (Table 2 and Figure 1 *b* and *c*). Supplementing the above medium with GA_3 (20 ppm) had significant effect on shoot proliferation as well as the number of shoots.

Table 1. Callus induction in scale explant of *Lilium polyphyllum* inoculated in MS medium supplemented with different concentrations of 2,4-D and cytokinins

Medium composition	% Callus induction in scale explants	Fresh weight (g)/culture
MS_0	–	–
2,4-D		
2.26	20.32	15.23 ± 0.9^{r}
4.52	45.56	28.25 ± 1.3^{q}
6.78	65.40	39.03 ± 1.5^{o}
9.05	59.97	36.28 ± 1.4^{p}
2,4-D + BAP		
6.78 + 1.33	75.21	107.17 ± 2.3^{e}
6.78 + 2.22	79.87	111.10 ± 2.4^{d}
6.78 + 3.11	85.21	130.95 ± 2.5^{c}
6.78 + 4.4	95.32	148.24 ± 2.6^{a}
6.78 + 6.6	89.22	133.22 ± 2.5^{b}
2,4-D + TDZ		
6.78 + 1.36	71.27	55.86 ± 1.7^{m}
6.78 + 2.27	78.55	71.54 ± 1.9^{k}
6.78 + 3.18	82.63	92.87 ± 2.1^{h}
6.78 + 4.54	90.07	103.95 ± 2.3^{f}
6.78 + 6.8	85.44	97.25 ± 2.2^{g}
2,4-D + kinetin		
6.78 + 1.39	69.38	48.33 ± 1.6^{n}
6.78 + 2.32	76.27	67.64 ± 1.8^{l}
6.78 + 3.25	80.00	87.44 ± 2.0^{j}
6.78 + 4.6	87.11	98.51 ± 2.2^{g}
6.78 + 6.9	81.46	89.27 ± 2.0^{i}

[#]Data are presented as mean ± SD. Means followed by different letters within a column indicate significant differences at $P \leq 0.05$.

The well-developed shoots were shifted to rooting medium in a cluster of 2–3 shoots. The shoots shifted to growth regulator-free medium showed 5% rooting frequency. However, incorporation of IBA (2.46–12.26 μM), NAA (2.65–13.25 μM) and IAA (2.85–14.27 μM) in MS medium of different salt strengths (full, half and quarter), resulted in an increase in rooting percentage. In addition to rooting, callusing was also observed at the excised end of shoots in normal MS medium. Since half-salt strength MS medium yielded better morphogenetic response, further experiments were conducted in this medium. Among different auxins used for root induction, IBA was the most effective followed by IAA and NAA respectively (Table 3). Half-strength MS medium enriched with IBA (9.8 μM) was found to be optimal medium for *in vitro* root induction, and 100% rooting was achieved with an average of 14.4 roots per shoot and 4.0 cm root length (Figure 1 *d–f*).

The *in vitro* raised plantlets with proper roots were shifted to plastic glass containing sterile soil and vermiculite in 1 : 1 ratio (Figure 1 *g*). Plants were kept inside the greenhouse for one month and then shifted to plastic glass containing compost-enriched soil. They were transferred to the polyhouse after one week (Figure 1 *h* and *i*). The plants were finally transferred to field with 85% success. Plants transferred to the pots started drying after sometime and simultaneously bulb formation took place. These bulbs started growing in the next season under conducive atmospheric conditions.

Human interventions have entirely transformed the land cover and land use, which are the key drivers of the loss of biodiversity[17]. Habitat destruction and unscrupulous overexploitation of plant species have resulted in the extinction of many species, and losing a species has grave ecological consequences[18]. *L. polyphyllum* is restricted to a few pockets in the North-West Himalaya, and overexploitation, habitat degradation and inflow of tourists during the period of its natural regeneration and flowering in the Himalayan region have led to considerable depletion of species from the wild. There is an ever-increasing demand of *L. polyphyllum* by the natural herbalists and herbal industry which cannot be fulfilled by the traditional methods of propagation. Hence, tissue culture

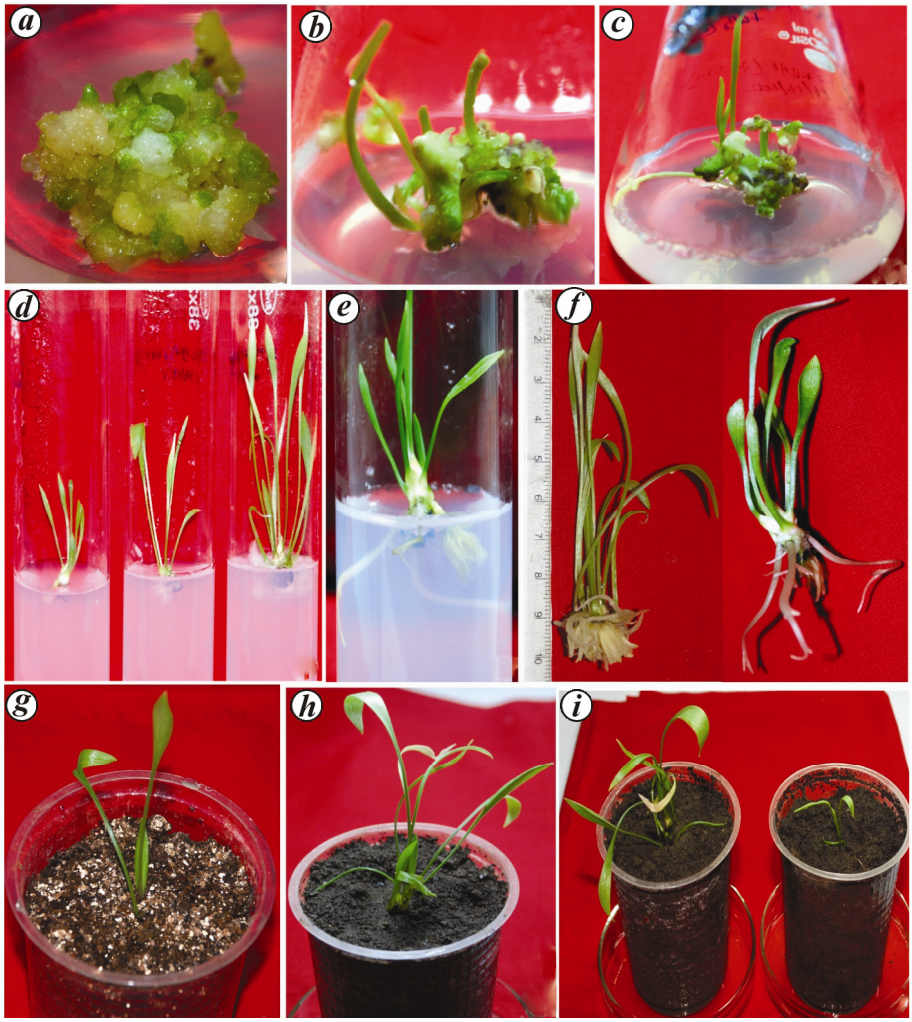

Figure 1. Micropropagation of *Lilium polyphyllum*: ***a***, Young callus showing shoot primordia. ***b***, ***c***, Callus showing shoot proliferation and well-developed multiple shoots. ***d***, Well-developed shoots inoculated in the rooting medium. ***e***, ***f***, Well-developed roots. ***g***, Hardening in vermiculite and soil (1 : 1 v/v) ratio. ***h***, ***i***, Hardening in compost-enriched soil.

technique offers tremendous potential for mass multiplication and conservation of the species.

Micropropagation via callusing has been used for mass multiplication of many species by applying different cytokinins and auxins. In the present study, 95.32% callusing was obtained in scale leaf explants inoculated in MS medium enriched with 2,4-D and BAP. However, Dhyani *et al.*[14] found only 69% callusing in MS medium fortified with 25 µM IBA. BAP and 2,4-D were also found suitable for the callus induction in *Lilium speciosum*[19]. The superiority of BAP over the other cytokinins, viz. TDZ and kinetin in callus induction has also been reported in *Rauwolfia serpentina*[20].

In the present study BAP was observed to be most effective in *in vitro* shoot induction in callus explants. The shoot formation was found 65% with mean shoot number and length of 10.6 and 6.1 cm respectively, in BAP. The superiority of BAP over kinetin has also been reported in *R. serpentina*[20,21]. The combined application of BAP and NAA was found best for multiple shoots induction and proliferation in callus explants and 97.45% shooting was achieved with average mean shoot number and shoot length of 19.2 and 8.6 cm respectively. This combination was also reported to be suitable for shoot induction in callus of *Habenaria edgeworthi*[22]. In the present study, TDZ had a negative effect on morphogenetic processes in callus explants. Similar findings have been reported for *Hypericum* species[23] and *Agastache rugosa*[24].

Hundred per cent rooting was achieved in half-strength MS medium enriched with IBA (9.8 µM), and IBA was observed as a potent root inducer in *L. polyphyllum* compared to IAA and NAA. The roots were better developed in half-strength MS medium in comparison to full strength MS medium. Nearly 85% survival rate was achieved after six weeks of transplantation in the field. The potted plants started drying after sometime and bulbs were developed as an underground part. These bulbs started growing under getting conducive atmospheric conditions.

Table 2. Effect of cytokinins and NAA on shoot induction in scale-derived callus of *L. polyphyllum* inoculated in MS medium after six weeks of culture

Cytokinin (μM)			Cytokinin + NAA (μM) + GA_3 (20 ppm)			Explants with shoots (%)	No. of shoots per explants	Shoot length (cm)
BAP	TDZ	Kinetin	BAP + NAA	TDZ + NAA	Kinetin + NAA			
MS_0	–	–	–	–	–	–	–	–
2.22	0	0	0	0	0	25.00	4.6 ± 1.6^{k}	4.0 ± 1.0^{l}
3.11	0	0	0	0	0	45.00	6.8 ± 1.9^{i}	5.5 ± 1.3^{i}
4.4	0	0	0	0	0	65.00	10.6 ± 2.5^{d}	6.1 ± 1.6^{g}
4.6	0	0	0	0	0	58.00	10.1 ± 2.5^{d}	6.0 ± 1.6^{g}
8.9	0	0	0	0	0	50.00	9.8 ± 2.5^{e}	5.9 ± 1.5^{h}
0	1.36	0	0	0	0	21.00	4.1 ± 1.6^{l}	3.9 ± 0.9^{m}
0	3.18	0	0	0	0	38.00	4.8 ± 1.6^{j}	4.8 ± 1.1^{k}
0	4.54	0	0	0	0	60.00	9.3 ± 2.5^{f}	5.8 ± 1.5^{h}
0	6.81	0	0	0	0	55.00	9.8 ± 2.5^{e}	5.5 ± 1.3^{i}
0	0	2.32	0	0	0	21.00	3.9 ± 1.3^{l}	3.9 ± 0.9^{m}
0	0	3.25	0	0	0	32.00	4.1 ± 1.6^{l}	4.1 ± 1.1^{l}
0	0	4.6	0	0	0	51.00	9.1 ± 2.3^{g}	5.9 ± 1.5^{h}
0	0	6.92	0	0	0	58.00	9.9 ± 2.5^{e}	5.6 ± 1.3^{i}
0	0	9.3	0	0	0	55.00	9.7 ± 2.5^{e}	5.3 ± 1.2^{j}
0	0	0	4.4 + 0.53	0	0	97.45	19.2 ± 3.6^{a}	8.6 ± 2.4^{a}
0	0	0	4.4 + 1.06	0	0	93.00	15.4 ± 3.1^{b}	8.4 ± 2.3^{b}
0	0	0	4.4 + 1.59	0	0	90.00	14.0 ± 3.0^{b}	8.1 ± 2.2^{c}
0	0	0	0	4.54 + 0.53	0	87.00	13.0 ± 2.8^{c}	8.0 ± 2.1^{d}
0	0	0	0	4.54 + 1.06	0	80.00	10.0 ± 2.5^{d}	7.9 ± 2.0^{e}
0	0	0	0	4.54 + 1.59	0	76.00	9.0 ± 2.3^{g}	7.5 ± 1.8^{f}
0	0	0	0	0	6.92 + 0.53	86.00	12.9 ± 2.8^{c}	8.0 ± 2.1^{d}
0	0	0	0	0	6.92 + 1.06	79.00	9.1 ± 2.3^{g}	7.9 ± 2.0^{e}
0	0	0	0	0	6.92 + 1.59	71.00	8.6 ± 2.0^{h}	7.6 ± 1.8^{f}

[#]Data are presented as mean ± SD. Means followed by different letters within a column indicate significant differences at $P \leq 0.05$.

Table 3. Effect of auxins on root induction in *in vitro* regenerated shoots of *L. polyphyllum* in half-strength MS medium after two weeks of culture

Auxins (μM)			Rooting (%)	No. of roots per shoot	Root length (cm)
IBA	NAA	IAA			
$1/2MS_0$	0	0	19	3.3 ± 0.1^{m}	2.0 ± 1.0^{j}
2.46	0	0	45	6.2 ± 0.6^{h}	2.3 ± 1.1^{h}
4.9	0	0	72	8.3 ± 0.9^{e}	3.0 ± 1.9^{f}
7.36	0	0	87	10.2 ± 1.3^{b}	3.9 ± 2.1^{a}
9.8	0	0	100	14.4 ± 1.6^{a}	4.0 ± 2.3^{a}
0	2.65	0	35	3.9 ± 0.2^{l}	2.1 ± 1.1^{i}
0	5.3	0	66	5.6 ± 0.4^{j}	3.0 ± 1.9^{f}
0	7.9	0	76	6.6 ± 0.7^{g}	3.2 ± 1.9^{e}
0	10.6	0	85	9.1 ± 1.0^{d}	3.6 ± 2.0^{c}
0	0	2.85	39	4.1 ± 0.2^{k}	2.8 ± 1.7^{g}
0	0	5.71	69	5.9 ± 0.4^{i}	3.2 ± 1.9^{e}
0	0	8.56	80	6.8 ± 0.7^{f}	3.4 ± 1.9^{d}
0	0	11.42	89	9.3 ± 1.2^{c}	3.8 ± 2.0^{b}

[#]Data are presented as mean ± SD. Means followed by different letters within a column indicate significant differences at $P \leq 0.05$.

In conclusion, a protocol has been standardized for the micropropagation of *L. polyphyllum* by organogenesis from *in vitro* regenerated callus. Micropropagation system assured effective propagation and mass multiplication of *L. polyphyllum*. It also offers an alternative for the conservation of this valuable, critically endangered and endemic herb of North-West Himalaya.

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Development of lifetime milk yield equation using artificial neural network in Holstein Friesian crossbred dairy cattle and comparison with multiple linear regression model

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The scope of this study was to develop lifetime milk yield (LTMY) prediction equation using different economical traits. The traits used were first lactation length, first peak yield, first lactation total milk yield, and total of three lactation milk yield of 1210 Holstein Friesian crossbred dairy cattle in India. Four variants of feed-forward back propagation algorithms were compared with the multiple linear regression model. The performance of Bayesian regularization (BR) algorithm was found to be better than the other algorithms for LTMY prediction. The BR neural network model was able to predict milk yield with 71.18% R^2.

Keywords: Artificial neural network, cows, lifetime milk yield, multiple linear regression.

ACCORDING to the National Dairy Development Board, India will need around 220 MMT milk by 2022. To achieve this target, we will need to improve productivity of our existing population of cows, as it is difficult to increase the number of animals due to shortage of land, feed and fodders[1]. In the last two decades migration of population towards urban areas has increased several fold in search of livelihood. So it is expected that the number of farmers will decrease and the number of animals per farmer will increase. In this situation, if a farmer could know how much milk his cow can produce in a lifetime, it will be the most important factor for further planning and management[2,3]. The analysis of lifetime milk yield (LTMY) is important for various reasons. It is helpful to select genetically superior bulls[4,5]. Milk yield prediction also helps in the selection of animals, which leads to optimal breeding strategies and increased annual genetic progress[6].

The objective of this study was to develop the LTMY prediction equation for dairy cattle. The cow starts giving milk after the birth of its calf; this is called the lactation period. Scientifically it should be 305 days to maintain the productivity of the animal. Standard lactation curve (Figure 1) follows a nonlinear pattern of milk production. Therefore, a nonlinear function should be used for the prediction of milk yield[7]. The traditional multiple linear regressions (MLRs) do not consider multicollinearity for

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